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Quantifying live potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) and cultural practices to reduce their survival

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Kwantificeren van levende aardappelcystenematoden (*Globodera rostochiensis* en *G. pallida*) en teelttechniekenom hun overleving te beperken

Illustration on the front cover: from left to right, a potato field; live eggs and a second-stage juvenile of potato cyst nematodes; white females and cysts on the roots and application of pig slurry in the field.

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Negin Ebrahimi

Ghent, 22nd of October, 2015

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List of abbreviations

PCN: Potato cyst nematodes

J2: Second-stage juvenile

J3: Third-stage juvenile

J4: Fourth-stage juvenile

PRD: Potato root diffusate

DD: Degree days

DD_4: Accumulated degree days above 4°C

DAI: Days after infestation

DAP: Days after planting

PP: Steamed potato peels

FPP: Fresh potato peels

L: Green leaves of leek

S+W: Soil and water

SCFA: Short chain fatty acid

PLFA: Phospholipid fatty acid

EC: Electrical conductivity

OC: Organic carbon

C:N: Carbon:nitrogen ratio

P-AL: Ammonium lactate extractable phosphorous

Ca-AL: Ammonium lactate extractable calcium

K-AL: Ammonium lactate extractable potassium

Mg-AL: Ammonium lactate extractable magnesium

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Summary

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida,* are significant threats to potato production worldwide. They have become a growing concern for potato producers due to the gradual withdrawal of chemical nematicides. The use of cultural methods can be one of the most environmentally sustainable strategies for limiting PCN damage and their spread. Second-stage juveniles of PCN can survive in soil for long periods of time as they are protected inside eggs within cysts. Therefore, in this research, I investigated the effects of cultural methods, *e.g.*, early harvesting of potato in early potato growing regions before the PCN life cycle is completed and the use of soil amendments on the survival and reproduction of PCN in the field. Recently, more attention is given to tare soil or soil adhering to tubers carried along with potato at harvest as an important means of PCN spread. Inundation of waste soil is an effective practice for the control of PCN; however, the process might take weeks to months. Therefore, I explored whether the effect of inundation on the survival of PCN in waste soil can be improved by amending locally available by-products from Belgian agro-industry. To determine the effects of amendments on the survival of PCN, expressed as the number of viable eggs, a quantitative viability assessing technique was needed. Hence, a quantitative technique was optimised based on trehalose present in the eggs as a viability marker.

To determine the effect of early harvesting on the survival of PCN, growth chamber, microplot and field trials were conducted to study the life cycle of PCN in growing conditions of early potato cropping. The development of populations of *G. pallida* on 3 cultivars, *viz.* Eersteling (susceptible to PCN), Première (resistant to *G. rostochiensis*) and Ambassador (partially resistant to *G. pallida*) and that of *G. rostochiensis* on cv. Eersteling was monitored in the growth chamber with simulated field temperatures of the season. *Globodera pallida* had a lower optimum temperature for hatching and developed faster than *G. rostochiensis*; its females appeared earlier than those of *G. rostochiensis*. On cvs Eersteling and Première, females and cysts of *G. pallida* populations were found 63 and 77 days after infestation with cysts (DAI), respectively. The number of degree days (DD) for *G. pallida* to complete its life cycle, using a base temperature of 4°C, was 450 DD_4. On cv. Ambassador, females of *G. pallida* were never observed. Females and cysts of *G. rostochiensis* were detected 70 and 84 DAI, respectively. This species needed 398 DD_6, at a base temperature of 6°C, to complete its life cycle. Observations in two fields and in microplots under prevailing weather conditions in 2013 revealed that cysts of *G. pallida* and *G. rostochiensis* were formed on June 12 and 15, respectively, when the

accumulated degree days were 463 DD_4 and 401 DD_6, respectively. These observations showed that both species of *Globodera* develop earlier than was assumed based on previous data (June 20).

To measure the viability of PCN, trehalose was extracted from cysts and from a dilution series of eggs, and quantified. More trehalose was detected when cysts were crushed than when left intact. Reaction volumes were adapted to the number of eggs because small reaction volumes hampered an accurate extraction of trehalose. A maximum of 10.5 eggs per µl reaction volume should be used to obtain a significant linear relationship between detected trehalose content and egg numbers. The sensitivity of the trehalose-based method was evaluated by determining the detection limit which was defined as 5 viable eggs. The reliability of this method was tested by comparing efficacy with that of two commonly used assays, visual assessment and hatching test. The trehalose-based method gave similar viability results as the visual assessment, but the latter is time consuming, requires trained personnel, and can involve some subjectivity. The hatching test identified fewer viable eggs than the other two methods. In addition, the viability of dead eggs (heated and naturally dead) was tested. No false positive results (dead eggs declared viable) were obtained with the trehalose-based method. The robustness of the test was demonstrated by measuring the viability of eggs of PCN in different experiments repeated in time. The viability assessment method based on trehalose proved to be an objective, sensitive, reliable, robust, fast, and cheap technique for assessing the number of viable eggs in PCN cysts.

I studied the effects of several amendments, *e.g.*, pig slurry, cattle slurry, mineral nitrogen fertiliser (NH_4NO_3), crab shell compost, wood chip compost and biochar (Romchar) alone or in combination with wood chip compost, pig slurry or crab shell compost, on the survival and reproduction of PCN in pot experiments. These amendments were mixed with a sandy soil and added to 2-L pots (survival test) or 4-L pots (reproduction test). Non-amended soil was used as a control. Cysts of *G. rostochiensis* or *G. pallida* were placed in retrievable nylon bags and added to the soil in each pot. Pots were left outside, exposed to prevailing temperature conditions, in a randomized design with 4 replicates. For the survival test, the viability of the cysts content was determined by visual assessment and by the trehalose-based method optimised in this research. Pig slurry, cattle slurry, mineral nitrogen fertiliser (NH_4NO_3), crab shell compost, wood chip compost and pig slurry mixed with biochar reduced the number of viable

eggs in cysts of both *Globodera* species in the absence of potato. The percentage mortality of encysted eggs due to the aforementioned amendments varied between 15 and 22.5% for *G. rostochiensis* and between 18 and 25% for *G. pallida*. Biochar alone or in combination with wood chip compost or crab shell compost, did not reduce the viability. When potato was planted in 4-L pots, less reproduction of both PCN species was observed in soil amended with pig slurry, wood chip compost, cattle slurry, nitrogen fertiliser, crab shell compost alone and mixed with biochar, compared with that in non-amended soil. The maximum reduction of the reproduction of both PCN species was achieved in soil amended with pig slurry (87%) and wood chip compost (82%). Biochar (Romchar) did not reduce the survival or reproduction of PCN. Moreover, it inhibited the suppressing effect of wood chip compost and pig slurry on PCN reproduction.

Amending soil with pig slurry, cattle slurry, nitrogen fertiliser, wood chip compost and crab shell compost inhibited the hatching, movement and root penetration of second-stage juveniles. A significant increase in the amount of ammonium was observed in the soils amended with nitrogen fertiliser (NH_4NO_3), pig slurry and cattle slurry. Phospholipid fatty acid analysis (PLFA) indicated an increase in the total biomass of the micobiota in soil amended with pig and cattle slurries, crab shell compost and wood chip compost. Therefore, these changes probably contributed to nematode suppression in the aforementioned amended soils.

I evaluated the possible added value of agro-industrial waste products on the suppressive effects of inundation on survival of PCN in waste soil. Agro-industrial waste products, *e.g.*, steamed and fresh potato peels and green leaves of leek were added to soil in inundated conditions. Calcium sulphate alone and mixed with steamed potato peels was also used in the experiment. PCN cysts were buried in the soil. The viability of the cysts content was determined at different time intervals after inundation using the trehalose-based method. Inundation of waste soil mixed with industrial waste products, such as steamed and fresh potato peels and leek for 4 weeks resulted in 99.9% reduction of viability of PCN cyst content, compared with 49% for non-amended soil. 72% egg mortality in non-amended inundated soil was obtained only after 8 weeks. Organic amendments reduced the pH and the redox potential of soil and increased the concentration of short chain fatty acids (SCFA). PLFA analysis also showed an increase in the

total biomass of microbial community of soils amended with potato peels. These changes can be associated with the suppressive effect of aforementioned amendments.

In conclusion, this research showed that non-chemical and cultural management strategies can lower the PCN populations in the field, provided they are applied wisely. Early harvesting at the right moment before the formation of cysts and choosing the appropriate amendments can reduce PCN survival and reproduction in the field. Inundation of PCN contaminated waste soil to which proper organic wastes were added also resulted in significant reduction of PCN survival. A good technique was also optimised to measure PCN survival (viability) in the laboratory.

Samenvatting

De aardappelcystenematoden (ACN), *Globodera rostochiensis* en *G. pallida*, zijn wereldwijd belangrijke bedreigingen voor de aardappelproductie. Ze zijn een toenemende zorg voor aardappeltelers als gevolg van de geleidelijke afschaffing van chemische nematiciden. Het gebruik van teeltmaatregelen kan een van de meest duurzame strategieën zijn om de schade veroorzaakt door ACN en hun verspreiding te beperken. ANC-juvenielen van het tweede stadium (J2) kunnen gedurende een lange periode overleven in de bodem omdat ze beschermd zijn binnen de eieren in de cysten. Daarom heb ik onderzocht welke de effecten van teeltmaatregelen (bv. vroeg oogsten van primeuraardappelen voordat de ACN levenscyclus voltooid is en het gebruik van bodemverbeteraars) zijn op de overleving en reproductie van ACN in het veld. Sinds kort wordt meer aandacht besteed aan losse aarde of aarde klevend aan geoogste knollen als belangrijk middelen voor de verspreiding van ACN. Het onder water zetten van deze restaarde is een doeltreffende praktijk voor de bestrijding van ACN; het proces duurt echter weken tot maanden. Daarom heb ik onderzocht of het effect van het onderwater zetten op de overleving van ACN in restaarde kan verbeterd worden door het toevoegen van lokaal beschikbare bijproducten van de Belgische agro-industrie. Om de effecten van bodemverbeteraars op de overleving van ACN, uitgedrukt als het aantal levensvatbare eieren, te bepalen, was een kwantitatieve techniek nodig om de levensvatbaarheid te schatten. Daarom heb ik een techniek, op basis van het gehalte van trehalose aanwezig in de eieren, geoptimaliseerd als merker voor de levensvatbaarheid.

Om het effect van een vroege oogst op de overleving van ACN te bepalen, werden groeikamer-, microplot- en veldproeven uitgevoerd om de levenscyclus van ACN, in groeiomstandigheden van vroege aardappelen, te bepalen. De ontwikkeling van populaties van *G. pallida* op 3 cultivars, nl. Eersteling (gevoelig voor ACN), Première (resistent tegen *G. rostochiensis*) en Ambassador (gedeeltelijk resistent tegen *G. pallida*) en dat van *G. rostochiensis* op cv. Eersteling, werd nagegaan in een groeikamer met gesimuleerde veldtemperaturen van het seizoen. *Globodera pallida* vertoonde een lagere temperatuur voor het uitkomen van J2 en ontwikkelde sneller dan *G. rostochiensis*; de vrouwtjes verschenen vroeger dan die van *G. rostochiensis*. Op cvs Eersteling en Première, werden vrouwtjes en cysten van *G. pallida* populaties gedetecteerd op respectievelijk 63 en 77 dagen na inoculatie met cysten (DNI). Het aantal graaddagen (DD) voor *G. pallida* om zijn levenscyclus te voltooien (basis temperatuur = 4°C) was 450 DD_4. op cv. Ambassador werden vrouwtjes van *G. pallida* nooit

waargenomen. Vrouwtjes en cysten van *G. rostochiensis* werden respectievelijk 70 en 84 DNI, gedetecteerd. Met een basistemperatuur van 6° C, had deze soort 398 DD_6 nodig om zijn levenscyclus te voltooien. Uit observaties in twee velden en in microplots, onder weersomstandigheden van 2013, bleek dat cysten van *G. pallida* en *G. rostochiensis* werden gevormd op 12 en 15 juni, resp; terwijl de geaccumuleerde graaddagen respectievelijk 463 DD_4 en 401 DD_6 waren. Beide *Globodera* soorten ontwikkelden zich eerder dan werd aangenomen op basis van eerdere gegevens (20 juni).

Voor het meten van de levensvatbaarheid van ACN werd trehalose geëxtraheerd uit cysten en uit een verdunningsreeks van eieren, en nadien gekwantificeerd. Meer trehalose werd gedetecteerd als cysten geplet werden dan wanneer ze intact werden gelaten. De reactievolumes werden aangepast aan het aantal eieren omdat kleine reactievolumes een nauwkeurige extractie van trehalose belemmerden. Maximum 10.5 eieren per µl reactievolume moet worden gebruikt om een significant lineair verband te verkrijgen tussen de gedetecteerde trehalose-inhoud en het aantal eieren. De gevoeligheid van de op trehalose gebaseerde methode werd beoordeeld door het bepalen van de detectiegrens; die werd gedefinieerd als 5 levensvatbare eieren. De betrouwbaarheid van deze methode werd getest door het vergelijken van haar werkzaamheid met die van twee andere veelgebruikte testen, nl. de visuele beoordeling en het uitkomen van de J2. De op trehalose gebaseerde methode gaf resultaten die vergelijkbaar waren met de visuele beoordeling; deze is echter tijdrovend, vereist opgeleid personeel en kan onderhevig zijn aan subjectiviteit. In de test van het uitkomen van J2 werden minder levensvatbare eieren geïdentificeerd dan met de twee andere methoden. Daarnaast werd de levensvatbaarheid van dode eieren (verwarmd en natuurlijk dode) getest. Met de op trehalose gebaseerde methode werden geen vals-positieve resultaten (dode eieren als levensvatbare verklaard) verkregen. De robuustheid van de test werd aangetoond door het meten van de levensvatbaarheid van eieren van ACN in verschillende experimenten herhaald in de tijd. De methodiek op basis van trehalose bleek een objectieve, gevoelige, betrouwbare, robuuste, snelle en goedkope techniek te zijn voor de beoordeling van het aantal levensvatbare eieren in ACN cysten.

Ik bestudeerde de effecten van verschillende bodemverbeteraars (varkensdrijfmest, rundveedrijfmest, minerale stikstof meststof (NH_4NO_3), krabschalencompost, houtsnipperscompost, biochar (Romchar) alleen of in combinatie met houtsnipperscompost,

varkensdrijfmest of krabschalencompost) op de overleving en reproductie van ACN in potexperimenten. Deze bodemverbeteraars waren gemengd met een zanderige bodem en toegevoegd aan 2-L potten (overlevingstest) of 4-L potten (voortplantingstest). De bodem zonder verbeteraars werd gebruikt als controle. Cysten van *G. rostochiensis* of *G. pallida* waren geplaatst in nylon zakjes en toegevoegd aan de grond in elke pot. Potten werden buiten, blootgesteld aan heersende temperaturen, geschikt in een gerandomiseerde opstelling met 4 herhalingen. Voor de overlevingstest werd de levensvatbaarheid van de cysteninhoud bepaald door visuele beoordeling en door de op trehalose gebaseerde methode geoptimaliseerd in dit onderzoek. Varkensdrijfmest, rundveedrijfmest, minerale stikstof meststof (NH_4NO_3), krabschalencompost, houtsnipperscompost en varkensdrijfmest gemengd met biochar, verminderden het aantal levensvatbare eieren in cysten van beide *Globodera* soorten in afwezigheid van aardappel. Het sterftepercentage van eieren in cysten als gevolg van bovengenoemde bodemverbeteraars varieerde tussen 18 en 22,5% voor *G. rostochiensis* en tussen 15 en 25% voor *G. pallida*. Biochar alleen of in combinatie met houtsnipperscompost en krabschaalcompost deed de levensvatbaarheid niet verminderen. Als een aardappelknol in 4-L potten geplant werd, werd in vergelijking met een niet verrijkte bodem, een geringere voortplanting van beide ACN soorten vastgesteld in bodems verrijkt met varkensdrijfmest, houtsnipperscompost, rundveedrijfmest en stikstof meststoffen, krabschalencompost alleen en gemengd met biochar. De grootste vermindering van de voortplanting van beide ACN soorten werd bereikt in de bodem verrijkt met varkensdrijfmest (87%) of houtsnippers compost (82%). Biochar (Romchar) heeft de overleving en reproductie van ACN niet verminderd. Meer nog, het remde het onderdrukkend effect van houtsnipperscompost en varkensdrijfmest op de ACN vermeerdering.

Het toevoegen aan de bodem van varkensdrijfmest, rundveedrijfmest, stikstof meststof, houtsnipperscompost en krabschalencompost remde het uitkomen van J2, hun verplaatsing in de bodem en de penetratie van de wortel. Een aanzienlijke toename van ammoniumnitraat werd waargenomen in de bodem verrijkt met stikstof meststof (NH_4NO_3), varkensdrijfmest en rundveedrijfmest. De fosfolipide vetzuuranalyse (PLFA) toonde een toename aan van de totale biomassa van de microbiota in de bodems met varkens- en runderendrijfmest, krabschaalcompost en houtsnipperscompost. Deze veranderingen hebben mogelijk bijgedragen tot de onderdrukking van nematoden in bovengenoemde verrijkte bodems.

Ik evalueerde ook de mogelijke toegevoegde waarde van agro-industriële afvalproducten op de onderdrukkende effecten van het onderwater zetten op de overleving van ACN in restaarde. Daartoe werden agro-industriële afvalproducten (bv. gestoomde en verse aardappelschillen, groene bladgedeelten van prei) alsook calciumsulfaat, alleen en gemengd met gestoomde aardappelschillen, toegevoegd aan onderwater gezette bodems. ACN cysten werden in de bodem ingebracht. Met behulp van de op trehalose gebaseerde methode werd de levensvatbaarheid van de cysteninhoud vastgesteld op verschillende tijdstippen na onderwater zetten. Inundatie van restaarde met agro-industriële afvalproducten zoals gestoomde en verse aardappelschillen en prei gedurende 4 weken, resulteerde in 99,9% daling van de levensvatbaarheid van de cysteninhoud; in niet-verrijkte bodems was dit 49%. In niet-verrijkt en onderwater gezette bodem werd pas na 8 weken 72% ei-sterfte vastgesteld. Organische verrijking verminderde de pH en redox potentieel van de bodem en verhoogde de concentratie van korte-keten vetzuren in de bodem. PLFA analyse toonde een toename in de totale biomassa van microbiële gemeenschap van bodems verrijkt met aardappelschillen. Deze veranderingen kunnen worden geassocieerd met het onderdrukkende effect van bovengenoemde bodemverrijkingen.

Kortom, uit dit onderzoek is gebleken dat niet-chemische en teelttechnische beheerstrategieën de ACN-dichtheden in het veld kunnen verlagen, op voorwaarde dat zij beredeneerd worden toegepast. Op het juiste moment (net voor cysten worden gevormd) oogsten en het kiezen van de juiste bodemverrijking verminderen het overleven en de voortplanting van ACN in het veld. Het onderwater zetten van met ACN gecontamineerde restgronden gemengd met plantaardig organisch afval heeft ook geleid tot een aanzienlijke vermindering van het overleven van ACN. Er werd ook een goede techniek ontwikkeld voor het meten van de levensvatbaarheid van PCN.

Chapter 1

General introduction

Potato (*Solanum tuberosum* L.) is the fourth-most important food crop in the world, next to rice, wheat and corn; it is the only tuber crop produced in a significant amount in temperate regions (Vreugdenhil *et al.*, 2007). Among the factors that adversely influence potato production, the potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, occupy a position of high economic importance (Haydock & Evans, 1998; Trudgill *et al.*, 2003). The annual loss they cause worldwide is estimated at approximately €300 million (Ryan *et al*., 2000; Deliopoulos *et al*., 2007). PCN have a remarkable ability to survive unsuitable conditions. Second-stage juveniles (J2) of PCN can survive in the soil for many years partially due to the physical protection provided by the eggshell and the robust cyst wall (Perry & Wharton, 1985; Jones *et al.*, 1998), but also because of their physiological adaptations, quiescence and diapause (Evans & Perry, 1976). The synchronisation of the PCN life cycle with the presence of a host plant also favours persistence of the nematode in the absence of the host in undesirable conditions (Perry, 1989). Soil temperature, soil moisture, soil structure, and photoperiod are also important environmental cues for the termination of dormancy resulting in hatching of J2 (Perry *et al*., 2013).

PCN are managed by integrating different control options, *viz.* crop rotation, nematicides and resistance (Anon., 2007; Suffert, 2014). In Belgium, early harvesting of potato is part of PCN management strategies. The legislation forbids farmers to enter potato in rotations shorter than 3 years, unless tubers are harvested before June 20 (Anon., 2010). Rotations are mandatory to prevent rapid build-up of potato cyst populations, but it is assumed that a short growing period at low soil temperatures (before 20 June) prevents the nematodes completing their life cycle, *i.e*., to produce eggs in new cysts. Yet, the assumption that early harvesting negatively impacts survival of the PCN population in the field might not be valid anymore because of *i*) climate change; *ii*) availability of new cultivars with new traits; *iii*) increasing prevalence of *G. pallida* and *iv*) the selection of virulent populations.

Annually, a large amount of agricultural and industrial by-products is generated worldwide. In Belgium, hog, poultry and cattle are the top-three livestock produced. A by-product of this livestock industry is manure and disposal of this waste is an issue. Animal wastes, pure as slurry or mixed with straw as manure, have been applied in the field as fertilisers for decades in Flanders. Agricultural crop production also leaves considerable amounts of waste.

Some of it is also recycled into the agricultural production as fertiliser, while large amounts remain unused. For instance, leek, one of the most important vegetable crops in Flanders, Belgium, is cultivated in different seasons throughout the year (Bernaert *et al*., 2012a; Vandewoestijne, 2014). The outer parts and the green leaves are the post-harvest crop wastes.

Previous studies have shown that different waste products, *e.g*., animal manures, crab shells, and several kinds of compost and plant extracts have the potential to manage plant-parasitic nematodes (Reynolds *et al*., 1999; Tian *et al*., 2000; Renčo *et al*., 2007; Xiao *et al*., 2008). However, there is limited knowledge concerning the effect of organic amendments, *e.g*., animal manures, composts and other agricultural waste products, including biochar, on the survival and reproduction of PCN and the mechanisms involved in nematode suppression. This information would help in the management of both PCN and agro-industrial disposal.

Potato cyst nematodes are mostly spread by soil adhering to farm machinery, moving PCN infested soils within and between fields, and by soil carried along with potato tubers at harvest (Goeminne *et al*., 2011). In Belgium, on average, 2.1 Mg soil ha^{-1} is taken from the field when harvesting potato (Ruysschaert *et al*., 2004). This tare soil, together with grading and sieving soil, collected after harvesting and potato processing, is considered as a waste product. Disposal of this waste soil is of major concern, especially when contaminated with PCN, a quarantine organism. Soil steaming (van Loenen *et al.*, 2003), soil fumigation and inundation (van Overbeek *et al.*, 2014) have been reported to be effective methods to kill potato cyst nematodes. However, steaming requires energy and most fumigants have been phased out because of concerns for human health and the environment; recently, the technique of covering soil with water, *i.e*. inundation, has received more attention (van Overbeek *et al.*, 2014).

To mitigate the damage caused by PCN in the field, the population levels should be kept as low as possible. Therefore, this research focused on several ways to reduce the cysts and the eggs and J2 contained within the cysts which are already in the field or might be spread into the field by tare soil. Hence, to reduce the survival of cysts content present in the field, potato can be harvested before the PCN life cycle is completed resulting in no formation of new cysts. Thus, the duration of the life cycle of the two PCN species was investigated on early potato cultivars in Belgian growing conditions. The reduction of PCN survival can also be achieved in the field in between potato crops by incorporating organic matters into soil. Therefore, the effect of agro-

industrial amendments - those locally available and economically acceptable by farmers, as well as new products like biochar- was determined on the PCN survival in between potato crops, as well as PCN reproduction when potato is planted. Finally, to prevent the introduction of cysts into fields by reducing the survival of PCN in waste soils, I explored whether the effects of inundation on the survival of PCN in waste soil can be improved by amending locally available by-products from Belgian agro-industry.

To determine the effects of amendments on the survival of PCN, expressed as the number of viable eggs, a quantitative viability assessing technique was needed. The viability of the PCN eggs can be measured by visual assessment based on egg and juvenile morphology, Meldola's blue staining followed by microscopic observations, hatching assays, or plant infectivity assays (OEPP/EPPO, 2013). Van den Elsen *et al*. (2012) reported a qualitative viability test based on the detection of trehalose in live eggs. This assay, however, was not applicable to enumerate live eggs.

Therefore, the specific objectives of this research were to:

1) Verify when the first cysts of *G. rostochiensis* and *G. pallida* appear on roots of early potato cultivars and determine the relationship between heat accumulation and PCN development in Belgian growing conditions.
2) Investigate whether potato cultivar or PCN population influence the time needed for cyst development.
3) Optimise a trehalose-based method to quantify live eggs of PCN, and evaluate the reliability and robustness of the method.
4) Evaluate the effect of different soil amendments on the survival of PCN in the absence of a host crop in pot experiments.
5) Examine the impact of the same amendments on the reproduction of PCN on potato in pot experiments.
6) Unravel the modes of action of these soil amendments in PCN suppression by investigating their effects on hatching, movement, host finding ability and root penetration of juveniles.
7) Evaluate the added value of agro-industrial waste products on the suppressive effects of inundation on survival of PCN in waste soil.

8) Investigate the mechanisms involved in this nematode suppression in inundated amended soils by determining the chemical and biological changes in these soils.
9) Understand the influence of amendments on PCN in inundated conditions by studying hatching, host finding ability and root penetration of the juveniles.

Chapter 2

Literature review

2.1 Origin, distribution and economic importance of potato cyst nematodes (PCN)

The potato cyst nematodes (PCN) include two closely related species, *viz. Globodera rostochiensis* (Wollenweber) Behrens and *G. pallida* (Stone) Behrens. They originated from South America and were introduced into Europe in the middle of nineteenth century (Evans *et al.,* 1975, Subbotin *et al.*, 2010), probably through the potato tubers imported to develop potato varieties resistant to potato late blight caused by *Phytophtora infestans* (Mai, 1977). PCN were then spread from Europe to different areas through seed potatoes (Hockland *et. al*., 2012). These nematodes have been reported on all continents where potatoes are grown (OEPP/EPPO, 2014a). PCN have been detected in 71 (*G. rostochiensis*) and 44 (*G. pallida*) countries (OEPP/EPPO, 2014a). Local dissemination of PCN within countries is largely by farm machinery and the contaminated soil attached to potato tubers. The survival strategies of PCN serve to aid their dissemination. Protected inside the eggs within the cysts, second-stage juveniles (J2) can remain dormant but viable for long periods of time up to twenty years (Jones *et al.*, 1998).

Potato cyst nematodes are among the major constraints to potato crop production and are the subject of strict regulations and quarantine measures in many countries. The annual loss they cause worldwide is estimated at approximately €300 million, about 12.2% of potato production (Ryan *et al*., 2000; Urwin *et al.*, 2001; Deliopoulos *et al*., 2007).

These nematodes have a limited host range including potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*) and some other *Solanum* species (Evans & Rowe, 1998). The symptoms of attack by PCN are not specific. Their primary effect on the host is the slowdown of root growth, which results in plants with smaller haulms, premature senescence and reduced tuber yield. Patches of poor growth occur generally in the crop. Stunting, yellowing, wilting or death of the foliage is sometimes associated with PCN infection (Turner & Evans, 1998).

Globodera rostochiensis has been the dominant species throughout most of potato growing regions where PCN are present. However, repeated growing of cultivars resistant only to *G. rostochiensis*, which has allowed the selective reproduction of *G. pallida* and the lack of nematicides with effective control over this species, led to an increase in the occurrence of *G. pallida* (Evans, 1993; Minnis *et al*., 2002).

2.2 Spread of PCN

Second-stage juveniles (J2) of PCN can only move over small distances; however, any means by which soil can be moved is considered a potential means of their passive spread (Jones, 1970). The PCN cysts can be spread by natural means, *e.*g., wind, water and animals or with humans. The local distribution of PCN (within and between fields) is attributed mainly to farming activities (Brodie & Mai, 1989). Sieving (tare) soil produced when the potatoes are taken from the harvester to the potato storage bins on the farm and sorting soil formed during packaging and transport can be contaminated with PCN when the potato lot originates from a PCN infested field. Therefore, movement of tare and sorting soil poses a high risk of spreading PCN over a short distance. Long-distance spread (within and between continents) is usually through the contaminated soil with PCN attached to seed potatoes and other planting materials (nursery stock and flower bulbs). PCN contaminated soil carried along with harvested potato tubers to potato processing factories and to other fields can be returned to a previously PCN-free area and contribute to the further spread of PCN. Movement of used machinery and the packaging materials can also cause the dispersal of PCN within a farmer's field, between fields of one farmer and from farm to farm, especially when some of the farm work is carried out by contracted labourers (Viaene *et al*., 2010; Goeminne *et al*., 2011; EFSA, 2012).

2.3 Life cycle of PCN

The life cycle of PCN includes the egg, four juvenile stages, and the mature adults. The first-stage juvenile (J1) moults to the J2 inside the egg. When stimulated by exudates from host roots, the infective J2 hatch from eggs and move towards the host root. They enter near the growing point or at emerging lateral roots by rupturing the epidermal cell walls with their stylet. Once inside the root, they move toward the vascular tissue through the internal cells (Evans & Stone, 1977) with the help of some degrading polysaccharides (*e.g*., β-1,4-endoglucanase), which contribute to cell wall degradation (Smant *et al.*, 1998). J2 ultimately establish a feeding site, *i.e*. a syncytium, become sedentary and develop to third (J3) and fourth (J4) stage juveniles and eventually to adults. The syncytia provide the developing J2 with a permanent food supply and play a role in sex determination. The J3 with large syncytia will develop into females, whereas those with small syncytia will either die or become males (Trudgill, 1967; Jones, 1970). The adult males leave the roots. The developing adult females swell and break through the

epidermis of roots, remaining attached but becoming spherical, with a projecting neck. The females release sex pheromones (Greet *et al*., 1968; Riga *et al*., 1996) and attract the adult males in the soil (Green *et al*., 1970). Males mate with the females, resulting in the production of 200-500 eggs retained inside the females' bodies. Eventually, females die and their cuticle forms a protective cyst around the eggs (Trudgill, 1967). After harvesting potato tubers, cysts detach from the roots and lodge in the soil. PCN can persist and survive for long periods of time as J2 inside the eggs within the cysts in the soil till the next host plant is grown (Turner & Evans, 1998). To assure the preservation of the species, the synchronisation between PCN and the host life cycle is important (Perry, 1997).

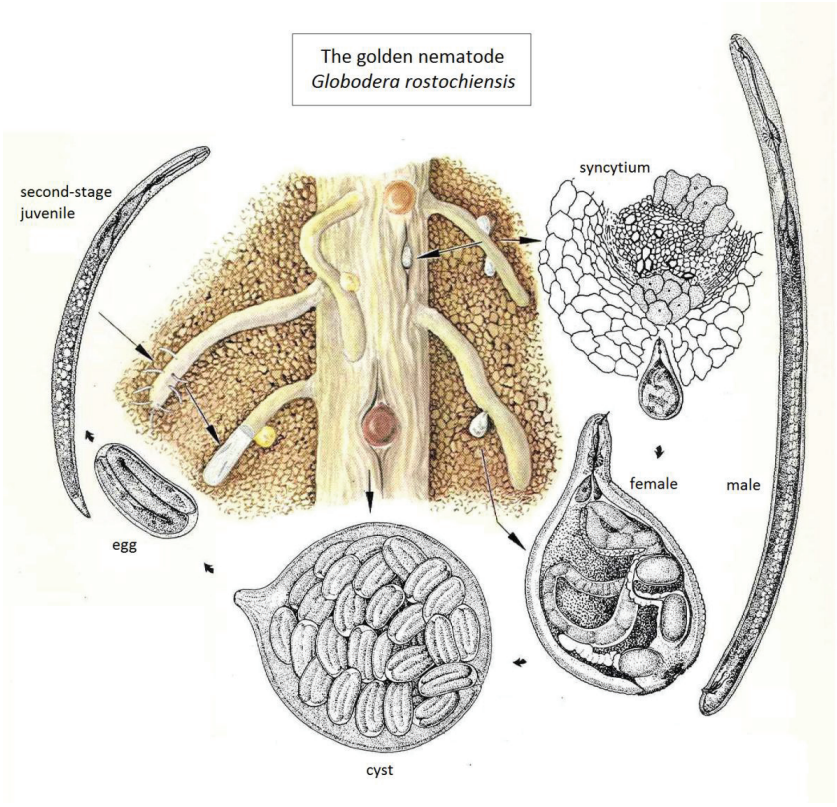


Figure 2.1. Life cycle of *Globodera rostochiensis*. Scheme after Charles S. Papp, CDFA, USA, with modifications.

The PCN life cycle is influenced by several factors including soil type, potato cultivar, fertilisation and other pathogens. Among all factors, temperature is of paramount importance (Franco, 1979; Trudgill *et al*., 2005; Van der Waals *et al*., 2013). Many plant-parasitic nematodes are adapted to particular temperature ranges and have different optimum temperatures for feeding, hatching, reproduction and survival (Neilson & Boag, 1996). Compared with *G. rostochiensis*, J2 of *G. pallida* hatch over a longer period and have a lower optimum temperature for hatching (Franco, 1979). It was shown that *G. pallida* females also develop faster and produce eggs earlier at lower temperatures (Webley & Jones, 1981).

2.4 Hatching of PCN

Hatching of PCN occurs as a response to host root diffusate (Perry, 1997), which ensures the emergence of J2 near the growing roots. However, it was shown that some J2 do not hatch even in close contact with the root diffusate, but need another exposure prior to hatching. It is assumed that this reduces competition for root penetration and feeding site formation (Perry, 1989). Perry (1986, 2002) proposed a bimodal action for the root diffusate hatching stimulus. Once unhatched J2 are exposed to host root diffusate, eggshell permeability is altered through a Ca^{2+}-mediated change in the inner lipoprotein membranes (Perry, 1986). The change in permeability results in the release of trehalose from the perivitelline fluid of the eggs and a consequent decrease in osmotic pressure on the unhatched J2. These J2 will be able to move and exit the eggs because of an increase in the water content as well as in the metabolic activities. These activities include increased O_2 consumption (Atkinson & Ballantyne, 1977a) and lipid utilisation (Robinson *et al.*, 1985), a fall in adenylate energy charge (Atkinson & Ballantyne, 1977b) and a change in cAMP levels (Atkinson *et al.*, 1987). It was also shown that exposure of *G. rostochiensis* to potato root diffusate had a stimulatory effect on the dorsal oesophageal gland with an increase in secreting granules (Atkinson *et al*., 1987; Perry *et al.*, 1989). J2 exit the eggs by a continuous thrusting movement of the stylet against the eggshell, which generates a slit (Perry *et al*., 2013).

2.5 Host localisation of PCN

The life span of J2 lasts 6-11 days after hatching under favourable environmental conditions (Robinson *et al*., 1987a). Therefore, once hatched, infective J2 must locate and move

towards host roots to complete their life cycle and have a successful invasion. It is known that the existence of gradients of amino acids, CO_2, sugars, ions and pH around the roots plays role in nematode attraction (Perry, 1997). Allelochemicals in the host root diffusate also attract J2 as these molecules interact with the amphidial secretions of J2 (Perry, 1997). However, it was shown that the chemicals in potato root diffusate stimulating hatching are different from those attracting J2 to roots (Devine & Jones, 2002). The orientation of J2 to the root tip is known, but the responsible active factors in the root diffusate are not yet recognised (Perry & Curtis, 2013). It is possible that specific allelochemicals in the root diffusate and/or an electrical potential gradient at the elongation zone of the root tip are involved in the local orientation (Balhadere & Evans, 1994). The role of chemoreception is evident as exposure of *G. rostochiensis* J2 to antibodies of amphidial secretions blocked the response to host root allelochemicals temporarily (Perry & Maule, 2004).

2.6 Survival of PCN

The life cycle of PCN consists of the pre-parasitic and the parasitic phase. PCN have some strategies to survive adverse environmental conditions at various phases of their life cycle. Understanding these survival strategies and factors involved in survival will help developing more effective management measures.

PCN have a remarkable ability to survive unsuitable conditions. After death of fertilised females, polyphenol oxidase tanning of the cuticle results in a hard, brown cyst surrounding the eggs. The juveniles inside the eggs rely on the physical protection provided by the eggshell and the trehalose content of the egg fluid, as well as the protective cyst for survival (Perry & Wharton, 1985). The unhatched J2 are able to suspend their development and remain in a dormant state inside the eggs within the cysts for up to twenty years (Hominick, 1986; Muhammad, 1994; Jones *et al.*, 1998) or until favourable conditions occur. The two forms of dormancy, quiescence and diapause, are physiological adaptations in PCN that are induced by environmental conditions (Evans & Perry, 1976). Quiescence is a reversible arrest in development induced in response to adverse conditions, whilst diapause is a state of arrested development, which cannot be resumed unless specific requirements have been satisfied, even in the presence of favourable conditions (Perry, 1997; Perry *et al*., 2013). Diapause is initiated by signals passed from the plant to the nematode during the growing season (Hominick *et al.*, 1985;

Hominick, 1986) in which the photoperiod affects the host plant and consequently, the hatch of the juveniles (Hominick, 1986). For example, the newly formed unhatched J2 of *G. rostochiensis* immediately enter obligate diapause. In this stage, temperature (a fixed period of cold temperature of autumn and winter) is an important factor in the termination of diapause (Perry *et al*., 2013). In early spring, the unhatched J2 enter obligate quiescence. This will be terminated by increasing soil temperature and by potato root diffusate if potato is planted. If no host is present, quiescence continues followed by facultative diapause as the non-stimulated eggs enter their second winter. This combination of diapause and quiescence enables PCN to persist in the soil for many years in the absence of the host plant (Perry *et al*., 2013).

The synchronization of host plant and the PCN life cycle favours persistence of the nematode in the absence of the host in undesirable conditions. However, every year, a small proportion of J2 spontaneously hatch in the absence of a host and die of starvation. This natural decline has been estimated approximately 20-40% per year for *G. rostochiensis* and 10-30% per annum for *G. pallida* (Whitehead, 1995). On average, *G. rostochiensis* has a 40% greater spontaneous hatch than *G. pallida* (Evans & Haydock, 2000). Schomaker & Been (2013) reported a mortality rate of 69% for *G. rostochiensis* in the absence of the host in the first year after a potato crop and 20-30% in subsequent years. The rate of this decline, however, depends on a number of environmental factors, such as soil type. In the field, 60-80% of J2 of *G. rostochiensis* hatch in the presence of host root diffusate, but some J2 will not hatch due to diapause and can remain viable in the field for several years (Turner & Subbotin, 2013). Therefore, the ability of PCN to be in a dormant state and to survive between successive crops, contributes significantly to PCN survival and dissemination.

Environmental factors affecting the survival of unhatched and hatched J2 of PCN include soil temperature, soil moisture, and oxygen concentration. There are reports on the effect of extreme temperatures on the survival of encysted eggs and J2 of PCN. It was reported that *G. rostochiensis* survived less than one hour when submerged in water warmer than 47°C (Wallace, 1963) or less than 3 min in 60°C aerated steam (van Loenen *et al*., 2003). Treating wet cysts with high temperatures (58-59°C) for 30 min led to the death of eggs and J2, whereas to kill eggs in dry cysts, 90°C for more than 30 min or 65-70°C for up to 24 h were required (Stone & Webley, 1975). Composting of organic wastes for 8 days at a minimum temperature of 50°C caused a

significant reduction in reproduction of *G. rostochiensis* (Boen *et al*., 2006). Unhatched J2 of *G. rostochiensis* survived freezing, either or not in contact with water; the eggshell and possibly the egg fluid surrounding the juveniles were accounted for such freezing survival. Hatched J2 in contact with water did not survive freezing while those not in contact with water, tolerated the sub-freezing temperatures (Perry & Wharton, 1985). Desiccation survival of unhatched J2 of *G. rostochiensis* was attributed to the protective function of the eggshell that reduced the rate of water loss from the J2 (Ellenby, 1968).

Spaull *et al*. (1992) showed that lack of oxygen in the soil caused a considerable decrease (99.3%) in the viability of encysted eggs and J2 of *G. pallida* after flooding contaminated soil for 14 weeks at 20 °C. Triffitt (1930) reported that *G. rostochiensis* J2 did not hatch in the absence of oxygen. No hatched J2 of beet cyst nematode, *Heterodera schachtii*, were observed in absence of oxygen, while the rate of J2 emergence increased with increasing oxygen concentration (Wallace, 1955). This author demonstrated that a brief exposure of *H. schachtii* cysts to low concentrations of oxygen (2 mg O_2 lit^{-1}) reduced the subsequent hatch of this species.

The hatched J2 are more vulnerable to environmental stresses and management strategies. These J2 rely on their lipid reserves until they penetrate the host roots. Robinson *et al*. (1987a, b) showed that the J2 of both PCN species remain infective in soil for relatively short periods. The reduction in the infection process (migration through the soil, the host root penetration and the feeding site formation) may be the result of depletion in energy reserves (Van Gundy, 1965; Robinson *et al*., 1987a). Hence, any management measure that aims at the stimulation of the hatch of PCN juveniles in the absence of a host crop can effectively reduce their population density in the field.

2.7 Management of PCN

Cyst nematodes are difficult to control because of their ability to persist in the soil without a host, their exponential rate of reproduction, and their protective cyst. Therefore, preventing the introduction and dissemination of PCN within a country and between countries is a necessary management strategy that should be considered by farmers and authorities. Once PCN become widespread, there will be significant costs for crop testing, nematicides, crop losses and export loss. It is evident that containment or eradication of *G. rostochiensis* can cost a

considerable amount before the benefits are achieved (Hodda & Cook, 2009). It should be noted that the management of *G. pallida* is even more challenging than that of *G. rostochiensis* because of its greater persistence in the soil with slower mortality rate (Whitehead, 1995), less effectiveness of nematicides against this species (Evans, 1993; Trudgill *et al*., 2014) and the absence of cultivars with complete resistance.

There are several management options to lower PCN populations below the damage threshold level or the population density above which the yield loss occurs: prevention, cultural practices (crop rotation, trap crops, and biofumigation), planting resistant and tolerant cultivars, biological control and nematicides (Roberts, 1993; Turner & Subbotin, 2013). Predictive software (NemaDecide) has also been developed as a grower aid to model the factors influencing yield losses and to make decisions on how to manage PCN (Been *et al*., 2005).

2.7.1 Prevention of PCN spread

Strict quarantine measures and the effective use of phytosanitary measures are in place to mitigate the introduction and eventually damage and spread of PCN. Pre-plant sampling and testing of fields for the detection of PCN is a requirement for seed potatoes. However, this is not in place for ware potato production due to difficulties in introduction of such regulation. Sampling soil attached to ware potatoes at loading or at import is an alternative to pre-plant soil testing. During sorting and packaging, the soil attached to tubers is dislodged. Soil samples can also be taken from bags and boxes containing tubers and tested. (OEPP/EPPO, 2014b). The farm machinery can be cleaned to reduce the amount of attached soil. In some countries, *e.g*., Germany, there is a national legislation based on which the farmers are not allowed to return the tare and sorting soil to agricultural fields (EFSA, 2012). Disinfestation of PCN contaminated soil can be a solution to prevent the distribution of PCN. The contaminated soil is taken to non-agricultural lands. This soil can also be disinfested by steaming, chemicals and inundation (van Loenen *et al.*, 2003; van Overbeek *et al.*, 2014).

2.7.2 Crop rotation

Crop rotation is an effective control measure to lower the PCN field population below the damage threshold levels (Riggs & Schuster, 1998) because of the restricted host range of PCN and the spontaneous hatch and the annual natural decline of PCN species. Crop rotation also

increases the diversity of the microbial community of the soil, such as antagonistic and predacious nematodes and other organisms that can control PCN (Nusbaum & Ferris, 1973; Widmer *et al*., 2002). It was reported that *G. rostochiensis* showing a greater spontaneous hatch than *G. pallida* is more readily controlled by crop rotation (Evans & Haydock, 2000). Trudgill *et al*. (2014) demonstrated that even if the annual decline of *G. pallida* were 33.5%, it would take 8 years for populations to decline to densities below damaging levels. In Europe, a crop rotation of 7 years without potato was recommended to reduce the PCN population in the soil to a significant degree (EPPO/CABI, 1997). When integrating the use of nematicides and resistant cultivars, this can be shortened (Phillips & Trudgill, 1998). However, crop rotation is limited in success because of the occurrence of other nematode species with wide host ranges, such as species of *Meloidogyne* or *Pratylenchus* (Viaene *et al*., 2013).

2.7.3 Resistant and tolerant cultivars

Resistant cultivars are considered the most effective environmentally friendly way to manage nematode populations, especially when they are used in combination with other control measures (Whitehead, 1998). Resistant cultivars allow little or no nematode reproduction, although plants may still suffer from the damage caused by nematodes at population densities above the tolerance limit. Resistant cultivars are cost effective, more environmentally friendly measure and can shorten the rotations (Trudgill, 1991). However, it should be noted that to date, only cultivars with complete resistance to *G. rostochiensis*, possessing the H_1 resistance gene, are commercially available. No single major gene resistant to *G. pallida* has been found, hence only partially resistant cultivars to this species have been developed (Trudgill *et al*., 2014); only limited numbers of these cultivars respond to expected quality and high yield. Partial resistance is defined as any resistance that causes less than 100% inhibition of nematode reproduction. In the Netherlands, the use of cultivars with partial resistance to *G. pallida* has been very successful. All starch potato cultivars are now partially resistant to *G. pallida*. There are also ware potato cultivars with highly partial resistance (> 99%) (EFSA, 2012). One has to consider that repeated cultivation of the same source of resistance to one PCN species will allow selective reproduction of another species (Evans, 1993). In the UK, *G. pallida* became a dominant species after repeated growing of the potato cultivar Maris Piper with the gene H_1 resistant to *G. rostochiensis* (Roberts, 1993; Trudgill *et al*., 2014).

It should be noted some potato cultivars are reported to be tolerant of PCN. Tolerant varieties usually have larger root systems and can withstand the damage caused by nematodes. As the volume of the root system available is larger, PCN have a lower yield-reducing effect on these cultivars than on a normal cultivar (Evans & Haydock, 1990). However, tolerant varieties are not useful for management of PCN as they permit the build-up of high nematode populations. Therefore, cultivars with both resistance and tolerance to PCN are recommended.

2.7.4 Trap cropping

Trap cropping is an alternative method of PCN control. A trap crop stimulates the hatch of PCN J2, while preventing their subsequent development or reproduction. Reproduction can be prevented by destruction of the crop before adult females have formed. Planting early potato cultivars and very early harvesting is a practice that decreases PCN populations (Webley & Jones, 1981), provided cysts have not formed. Scholte (2000a) showed that planting susceptible potato as a trap crop can reduce the nematode population. However, timing of crop destruction should be very precise not to increase the PCN population in the field instead. A safe procedure to apply trap cropping is to identify the PCN species in the field and plant a resistant cultivar. Tubers should be planted at a high density so that many roots develop and can stimulate hatch of as many juveniles as possible (Viaene *et al*., 2013). An average reduction of 95% can be obtained with a resistant potato trap crop (EFSA, 2012). Another safe procedure for trapping of PCN is to use a non-host crop such as *Solanum sisymbriifolium*, which stimulates hatching of *G. pallida* by 77% and does not allow its reproduction. This plant decreased PCN populations by more than 50% in severely infested fields (Scholte, 2000b; Scholte & Vos, 2000). However, Hartsema *et al*. (2005) found a highly variable efficacy of this trap crop. The average population decline of PCN in the field planted with *S. sisymbriifolium* was 52%, little more than the average natural decline in the tested fields, and much lower than that in the fields with a resistant potato trap crop. Halford *et al*. (1999) found that trap cropping with potato was time-consuming and labour intensive with few immediate benefits for the grower.

2.7.5 Biofumigation

A potential approach to manage PCN is biofumigation. This method consists of crushing and incorporating plant materials (*e.g*., green manures) containing organic compounds called

glucosinolates into the soil. The efficacy of this method can be improved by tarping the soil with plastic foil or soil compaction (Ploeg, 2008; Thoden *et al*., 2011). Recent studies have demonstrated the potential of brassicaceous crops to control *G. pallida* in potato production (Valdes *et al*., 2011; Lord *et al*., 2011; Ngala *et al*., 2015). Incorporation of *Brassica juncea* leaf tissue into soil caused 85-95% mortality of encysted eggs of *G. pallida* in uncovered and polyethylene-covered potted soil, respectively (Lord *et al*., 2011). The suppressive effect of brassicaceous residues is attributed to toxic volatile isothiocyanates and toxic organic cyanides, oxazolidinethiones, nitriles and ionic thiocyanate released through enzymatic hydrolysis of glucosinolates by myrosinase (Bones & Rossiter, 2006). Ngala *et al*. (2015) reported positive relationships between the glucosinolate concentration and the mortality of *G. pallida* assessed 6 weeks after incorporation of the brassicaceous residues.

2.7.6 Biological control

Biological control can also be considered one of the options to manage PCN. This control is mainly based on the interactions between PCN and predator nematodes, fungi, bacteria and mycoplasma-like organisms (Ferris *et al*., 1992; Khan & Kim, 2007). Since PCN eggs inside cysts remain for long periods in the soil, they can be a target for biocontrol agents (Riggs & Schuster, 1998). Penetration of potato roots by *G. pallida* was reduced by the systemic resistance in the potato induced by the rhizobacteria *Agrobacterium radiobacter*, *Bacillus sphaericus* or *Rhizobium etli* (Hasky-Gunther *et al*., 1998; Reitz *et al*., 2000). So far, few organisms have been developed as practical agents and none is widely used. Tobin *et al*. (2008) demonstrated the efficacy of *Pochonia chlamydosporia* as a biological control agent of PCN and indicated its potential for use as part of an integrated pest management strategy. Except for microbial-induced suppression of nematode pests, biological control agents provide too little control to be effective alone. Therefore, their successful use in sustainable management strategies will depend on their integration with other control measures (Viaene *et al*., 2013).

2.7.7 Chemical control

Nematicides have provided a very effective management of PCN (Whitehead, 1998) but many chemicals are now restricted or forbidden because of their potential health and environmental risks (Rich *et al*., 2004). Moreover, the effectiveness of nematicides can be influenced by the

environment (*e.g*., temperature), soil properties and soil microbial activities, which can increase the degradation of nematicides (Smelt *et al*., 1987; Haydock *et al*., 2013). In addition, nematicides are expensive for the farmers.

2.7.8 Amendments

Amending soil with various sources of organic matter has led to reduced disease incidence caused by plant pathogens and pests either by reducing population levels or by increasing yields without affecting populations (Cook, 1986; Abawi & Widmer, 2000; Akhtar & Malik, 2000; Conn & Lazarovits, 2000; Gamliel *et al*., 2000; Lazarovits *et al*., 2001; Widmer *et al*., 2002).

Nematode suppression by soil amendments is caused by different mechanisms including *i*) the release of nematicidal compounds present in soil amendments or generated during their degradation, *e.g*., ammonia and fatty acids *ii*) increase in plant tolerance, *iii*) improvement of soil physical and chemical properties which may have an adverse influence on hatching, mobility and survival of nematodes (Oka, 2010) and *iv*) introduction of microorganisms beneficial for plant growth or antagonistic towards nematodes (Stirling, 1991; Viaene & Abawi, 2000; Arancon *et al.,* 2003).

The most common amendments are wastes or by-products of agricultural industries, such as animal manures, compost and plant residues (Oka, 2010). Although such waste products tend to be inexpensive, large quantities of them must be added to the soil to be effective; hence, they must be locally available.

2.7.8.1 Animal manures

Applying animal manure is a regular practice for the improvement of the soil fertility in many agricultural systems. Therefore, the additional benefit of nematode management may be a useful supplementary gain. The mechanisms of nematode suppression by animal manures have been investigated but appear complex. Apart from the toxic compounds generated by manures in soil such as ammonia and volatile fatty acids, nematode suppression may also be caused by changes in the soil microfauna and microflora and the increase of the populations of antagonistic microorganisms to nematodes. Kaplan & Noe (1993) demonstrated a linear relationship between

the decrease in population density of *M. incognita* and an increase in number of bacteria by increasing rates of chicken litter in the soil. Reynolds *et al*. (1999) and Xiao *et al*. (2008) reported the effective management of plant-parasitic nematodes by swine manure.

2.7.8.2 Composts

Composts are generally derived from agro-industrial wastes, such as tree bark, animal and green wastes; they provide the soil with nutrients and organic matter and improve the soil structure. Composts are known to be effective in the control of soil-borne pathogens such as *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia* (Hoitink *et al*., 1997). Some antagonistic organisms *e.g*., fungi (*Trichoderma*, *Penicillium* and *Aspergillus*), bacteria (*Bacillus*, *Pseudomonas* and *Pantoea*) and actinomycetes are thought to be responsible for disease control (Hoitink & Boehm, 1999). Some of these microbial genera are also known to be suppressive to nematodes (Kluepfel *et al*., 2002; Kokalis-Burelle *et al*., 2003; Mekete *et al*., 2009). Various reports indicated that certain composts and mulches reduced population levels of plant-parasitic nematodes. Composts and raw sewage sludge showed activity against root-knot and root-lesion nematodes (Marull *et al*., 1997; LaMondia *et al*., 1999; Everts *et al*., 2006). Lazarovits *et al*. (1999) found that populations of root-lesion and root-knot nematodes were reduced already two weeks after the addition of soybean or meat bone flour to commercial potato fields in Ontario. However, the effect of compost and other amendments is variable and often conflicting. In some studies, composts did not have a significant suppressive effect on nematodes (McSorley & Gallaher, 1995, 1996). Likewise, in a long-term experiment, application of compost increased total yields of potato tubers and grain yields of barley, but the populations of *Pratylenchus penetrans*, *M. hapla* and *Heterodera trifolii* were usually increased (Kimpinski *et al*., 2003). The mechanisms of nematode suppression by composts have not been studied in details. Two studies indicated that nitrogenous compounds, rather than microorganisms, contribute to the nematode suppression (Oka & Yermiyahu, 2002; Raviv *et al*., 2005).

2.7.8.3 Chitinous amendments

Chitinous materials have received special attention for their suppressive effects on plant-parasitic nematodes. Two major mechanisms involved in nematode suppression by chitinous amendments are *i*) the generation of toxic nitrogenous compounds, mainly ammonia and nitrous

acid, soon after the amendment application (Spiegel *et al*., 1987) and *ii*) the increase in number and activity of chitinolytic microorganisms, such as bacteria, fungi and actinomycetes, as well as chitinase activity in the soil a few months after soil amendment with these materials, leading to degradation of chitin-rich eggshells of nematodes (Mian *et al*., 1982; Rodríguez-Kabana *et al*., 1987; Spiegel *et al*., 1987; Hallmann *et al*., 1999; De Jin *et al*., 2005; Kielak *et al*., 2013). Even though the mechanism of control is not fully understood, the addition of crustacean chitin was proven highly effective, leading to the registration of commercial products.

Cronin *et al*. (1997) reported that purified commercial chitinase inhibited the hatching of *G. rostochiensis*, *in vitro* up to 70%. The chitinase produced by *Paenibacillus illinoisensis* KJA-424 also caused the lysis of *M. incognita* eggshells, resulting in 100% inhibition of hatching *in vitro* (Jung *et al*., 2002). The incubation of *M. hapla* with the chitinase derived from bacteria resulted in early emergence of J2 and increase in the hatching rate (Mercer *et al*., 1992). Tian *et al*. (2000) found that soil amendment with chitin may improve the development of nematode antagonistic soil fungi and bacteria, leading to suppression of *H. glycines* population densities.

In general, application of organic soil amendments is a traditional management strategy and it is considered a part of plant-parasitic nematode-management programs. Furthermore, combination of soil amendments with some other management strategies *e.g*., solarisation (Butler *et al*., 2012) or flooding (van Overbeek *et al*., 2014) has provided more effective control of plant pathogens including nematodes.

Chapter 3

General materials and methods

3.1 Potato cyst nematode cultures

Cysts of one population of *Globodera rostochiensis* (Kruishoutem, East Flanders, Belgium) and of two populations of *G. pallida* (Chavornay, Switzerland (the reference population for official potato resistance testing (Anon., 2007)) and GOV 038, East Flanders, Belgium) were obtained from stock cultures. These populations were maintained on potato (*Solanum tuberosum*) cv. Désirée. Plants were grown in 3-L pots filled with a growing medium composed of silver sand, clay powder and expanded clay granules (4:0.7:1; w/w/w) (Seinhorst, 1984). This medium facilitated the extraction of PCN cysts using a Seinhorst elutriator (Seinhorst, 1964). One sprouting potato tuber was planted per pot and left to produce roots during about 10 days. Then, the substrate was infested with 5 eggs and J2 per cm^3 soil and cultures were maintained in a greenhouse (20-25°C, 16 h light) and watered regularly. After sixteen weeks, plants were uprooted and the substrate was collected in plastic bags. The substrate was stored at 4°C when the cysts extraction was not performed immediately.

Newly formed cysts were extracted from the substrate using a Seinhorst elutriator (Seinhorst, 1964). Cysts were kept dry in vials for at least 4 months at 4°C to overcome diapause before use in experiments. The average number of eggs per cyst was based on a subsample of 20 cysts per batch of new cysts. Cysts were placed in a drop of water in a Petri dish and crushed. The released eggs were rinsed into a beaker over a 60 µm sieve and the volume adjusted to 50 ml. The number of eggs and J2 in three 3 ml subsamples was determined using a microscope (100×).

3.2 Extraction of nematodes from soil

3.2.1 Extraction of cysts

The cysts obtained from pots, microplots or fields were extracted using a Seinhorst elutriator (Seinhorst, 1964) (Figure 3.1). Soil with cysts was poured on a sieve placed on top of the funnel shaped elutriator. The flow rate of an upward current of water was adjusted and maintained at 3 L min^{-1} throughout the whole extraction process. This upward water flow allowed the heavier soil particles to settle while retaining the cysts and smaller particles in the funnel. The small and light cysts floated and were washed into a 250-µm sieve fitted into a bucket. The heavier full cysts floated in the upper half of the funnel and passed through a side

outlet and were directed into the same sieve. The content of the bucket was rinsed over an 850-µm sieve placed on top of a 250-µm sieve. The cysts and small debris were collected in the latter sieve and kept at room temperature for at least 24 h to dry. Once the organic debris was dry, the cysts were picked out and counted.

Figure 3.1. Seinhorst elutriator (Courtesy of ILVO, Merelbeke).

3.2.2 Extraction of *Globodera* free-living stages

The automated zonal centrifuge (AZC) (Hendrickx, 1995) (Figure 3.2) was used for the extraction of free-living stages of potato cyst nematodes (PCN) from the soil. A sample of 200 cm^3 soil was washed through an 850-µm sieve placed on a 1000-ml plastic beaker to separate the organic fraction from the mineral part. The organic fraction was collected from the sieve and blended at high speed for 1 min before it was mixed with the mineral fraction in the beaker. The sample was then processed in the AZC. The volume of the solution containing the mineral and organic fraction was first increased to 1 L. Then, a 500-ml subsample was gradually added to two layers with different densities formed inside a spinning centrifuge (rotor). The nematodes were separated from the soil particles and moved to a layer with density between 1 (water) and

1.20 ($MgSO_4$). When the spinning gradually stopped, the sediment was sealed off with a layer of kaolin and the layer with the nematodes was removed through the hollow shaft of the rotor. The clear nematode suspension of about 200 ml was then collected in a beaker and set aside to allow nematodes to settle to the bottom. After removing the supernatants, the remaining nematode suspension of about 40 ml was transferred to a counting dish for counting and identification of nematodes. As the AZC only processed half of the sample (500 ml), the number of nematodes corresponded with 100 cm^3 soil.

Figure 3.2. Automated zonal centrifuge machine.

3.3 Collection of potato root diffusate

Potato root diffusate (PRD) was prepared according to a modification of the protocol described by Turner *et al*. (2009). A potato tuber cv. Désirée was planted in a 2-L pot filled with a sterilised sandy soil. After 4 weeks, the soil was saturated with water and additional 100 ml water was added. The solution draining from the pot was collected and passed through the pot again. The second soil leachate, containing the root diffusate was collected and filtered (Munktell filter paper no. 2601, 150 mm, basis weight 40 g m^{-2}, Munktell Filter AB, Falun, Sweden) to remove soil particles. The root diffusate was stored at -20°C for later use.

3.4 Hatching assay

Cysts of *G. rostochiensis* or *G. pallida* were soaked in tap water for 48 h before they were exposed to PRD. Cysts were transferred into 1.5-ml Eppendorf tubes with the centre of the lid replaced by a 250-µm sieve made of nylon voile and the constricted end of the tubes cut off. These tubes were then transferred to 15-ml plastic bottles. Two ml of the test solution was added to the bottles just touching the sieve. The samples were maintained in dark at 21°C in a completely randomized design. The number of hatched J2 was counted weekly over a period of 8 or 10 weeks (varying in different experiments); the solution was renewed at every counting event. After 8 or 10 weeks, all cysts were crushed and the number of unhatched juveniles inside the cysts was determined to calculate the percentage of the total cyst content that had hatched during the assay.

3.5 Infectivity test in the closed container

To evaluate the ability of PCN J2 to penetrate the potato roots, an infectivity test was conducted in 0.5-L closed plastic containers (11 cm diam., 8 cm height). Tubers of potato cv. Désirée were first disinfested with sodium hypochlorite (NaOCl) (5%) for 4 min and kept at room temperature to sprout. When sprouts were visible, tubers were planted in closed containers filled with 200 g sterilised river sand (0.5 mm diam.) and 30 ml tap water and kept in the dark at 21°C for 1 week. When the roots had formed, the sand was infested with cysts or J2 of PCN. Sand was used to allow easy removal of roots at harvest. Containers were kept in dark at 21°C. Four weeks later, roots were gently removed, washed and juveniles inside the roots were stained and counted.

3.6 Staining nematodes inside the root

The nematodes inside the root tissues were stained following the method described by Byrd *et al.* (1983). Roots were washed carefully and cut into pieces of 1-2 cm. They were placed in a beaker and covered with 1% NaOCl for 4 min with occasional agitation. Then the root segments were placed on sieves and rinsed with tap water to remove the NaOCl. Roots were transferred into a beaker containing 30 ml of distilled water and 1 ml of staining solution (3.5 g acid fuchsin, 250 ml acetic acid and 750 ml water) and boiled for 30 s. After the liquid with roots cooled down, roots were rinsed with water, transferred to a beaker, covered with glycerin and

boiled to destain the roots. After the samples had cooled, the nematodes inside the root segments were observed using a microscope.

3.7 Statistical analysis

The data obtained from each experiment were subjected to ANOVA. Data were transformed when the assumptions of normality and homogeneity of variances were not fulfilled. The non-transformed data were used to draw the graphs. The differences among the treatments were analysed with Tukey's Honestly Significant Difference (HSD) test and differences were considered significant when $P < 0.05$. The data from the repeated experiments were combined and used in a single analysis when these data did not differ significantly. These analyses were performed with STATISTICA_9 and STATISTICA_10 software (Statsoft, OK, USA).

Chapter 4

Observations on the life cycle of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, on early potato cultivars

Ebrahimi, N., Viaene, N., Demeulemeester, K. & Moens, M. (2014). Observations on the life cycle of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, on early potato cultivars. *Nematology* 16, 937-952.

4.1 Introduction

Nematodes are preferentially managed by integrating several control strategies. Within the strategies used to control PCN, very early harvesting of early potato cultivars is an elegant practice that decreases the survival of PCN populations (Webley & Jones, 1981) because the short growing period at low soil temperatures reduces the time available for the nematodes to complete their life cycle and to produce new cysts.

By entering diapause and quiescence when inside the cyst, second-stage juveniles (J2) of PCN can survive for long periods of time (Grainger, 1964). Eventually they hatch from the egg under the stimulus from both biotic and abiotic factors. Potato root diffusate is a biotic factor of paramount importance; in the absence of root diffusate J2 will hatch only in very limited numbers (den Ouden, 1960). To assure the preservation of the species, the synchronisation between PCN and the host life cycle is important (Perry, 1997). Among the abiotic factors, temperature is the most important environmental cue for the termination of the obligate diapause and quiescence of PCN (Perry *et al.*, 2013). High soil temperatures accelerate the development of PCN; below a threshold, *i.e.*, the base temperature, development is stopped. However, because of the high variation in temperature from location to location and year to year, calendar-based scheduling for nematode development and timing management practices may not be accurate. Yet, when comparing different systems for predicting temperature-related insect population and crop development, Wilson & Barnett (1983) demonstrated that the amount of heat an organism needs to complete its development, *i.e.*, the combination of temperature and time, is constant. The measure of accumulated heat, known as physiological time, is expressed in degree days (DD). The factor temperature in the calculation of the number of degree days is usually determined as the mean daily temperature minus the base temperature needed for development of the organism. However, there are a number of ways to calculate DD, *e.g.*, the averaging (*i.e.*, rectangle) method, sine-wave approaches and three triangulation approaches (Roltsch *et al.*, 1999).

For *G. rostochiensis* and *G. pallida* the base temperature was established at 5.9°C and 3.9°C, respectively (Mugniéry, 1978; Langeslag *et al.*, 1982). Alonso *et al.* (2011) investigated the relationships between the heat accumulation and the development of *G. pallida*. Their study showed differences in the pattern of occurrence of developmental life stages on two potato

cultivars susceptible to both *Globodera* species. Compared with the late cv. Marfona, *G. pallida* required at least 100 DD more on the early potato cv. Maris Peer to reach the adult stage. It has also been shown that the two PCN species are noticeably different in their behaviour and life cycle (Den Nijs & Lock, 1992). Compared with *G. rostochiensis*, *G. pallida* has a lower optimum temperature for hatching (Franco, 1979) and its females develop faster and produce eggs earlier at lower temperatures (Webley & Jones, 1981). The latter authors also observed a significant population decline of both species after an early harvest of early potatoes. Previous research also showed that under field conditions, *G. rostochiensis* can adapt to hatch better at low temperatures than under controlled conditions (Ellenby & Smith, 1975; Hominick, 1979).

In Belgium, The Netherlands, UK (Pickup *et al.*, 2012) and Norway (Holgado & Magnusson, 2010), early harvesting of potato is part of PCN management strategies. In Belgium, this legislation allows the farmers to grow potato in rotations shorter than 3 years. Tubers should be harvested before June 20 (Anon., 2010) because it is assumed that no cyst formation occurs by that date. However, this assumption might not be valid anymore because of *i*) climate change; *ii*) availability of new cultivars with new traits; *iii*) increasing prevalence of *G. pallida* and *iv*) selection of virulent populations. In view of these changes, the objectives of this study were to verify when cysts of *G. rostochiensis* and *G. pallida* appear on early potato varieties and to determine the relationship between heat accumulation and the development of the two PCN species in Belgian growing conditions. The influence of the potato cultivar and the PCN population was also investigated on the time needed for cyst development. Pot experiments were conducted in the growth chamber to study the life cycle of different PCN populations on three potato cultivars. To obtain a realistic view on the *Globodera* life cycle, the occurrence of developmental stages of the nematode was also monitored on potato grown outside under prevailing climatic conditions in two infested fields kept under common farming practices and in artificially infested microplots.

4.2 Materials and methods

4.2.1 Nematode populations and plant materials

Cysts of one population of *G. rostochiensis* (Kruishoutem) and of two populations of *G. pallida* (Chavornay, highly virulent (Anon., 2007) and GOV 038) were obtained from stock

cultures maintained on potato cv. Désirée under glasshouse conditions (20-25°C, 16 h light). After their extraction from the soil (Seinhorst, 1964) (see section 3.2.1), cysts were kept at 4°C for 4 months to overcome the diapause of the J2 before use in the experiments. The three PCN populations were used in the growth chamber experiments. The soil of the microplots was infested with either *G. rostochiensis* Kruishoutem or *G. pallida* GOV 038. The soil of the fields in Roeselare and Tielt were naturally infested with *G. pallida* (Table 4.1). Potato cultivars used were Eersteling, Première, Ambassador, Sinora and Lady Christl (Table 4.1).

Table 4.1. Overview of experiments, showing populations of *Globodera* species and potato cultivars.

	G. rostochiensis	***G. pallida***			
Population	**Kruishoutem**	**GOV 038**	**Chavornay**	**Roeselare**	**Tielt**
Cultivar/Growth chamber	Eersteling	Eersteling	Eersteling		
		Première			
		Ambassador			
Microplot	Eersteling	Première			
Field				Sinora	Lady Christl

4.2.2 Growth chamber experiment

Pots of 2.5 l filled with sterilised loamy sand soil were planted with a single tuber of one of the following early potato cvs: Eersteling (susceptible to both *G. rostochiensis* and *G. pallida*), Première (resistant to Ro1) and Ambassador (partially resistant to Pa2 and Pa3) (Table 4.1). At planting, only one sprout was left on each tuber to obtain a root density which can be representative for a field situation. The same day, batches of 15 cysts were prepared of each PCN population and placed in retrievable bags made of nylon voile, which were added to the soil at a depth of 6 cm; based on the average content of the cysts, the initial density was 2.7 eggs $(cm^3$ soil$)^{-1}$. Pots planted with cv. Eersteling were inoculated with one of the three PCN populations (*G. rostochiensis* Kruishoutem, *G. pallida* Chavornay and *G. pallida* GOV 038); pots with cvs Première and Ambassador were inoculated with *G. pallida* GOV 038 only. A total of 56 pots for each of the five combinations of nematode population and cultivar were placed in a growth chamber. Both the diurnal temperature and the day length were set weekly (*e.g.*, in week 2, day

temperature was set at 8°C during 12 h and 20 min and night temperature at 3°C during 11 h and 40 min) following the 6-year weather statistics of the growing season of early potatoes in Belgian production regions (PCA-Kruishoutem) (Figure 4.1). The time lag between air temperature and soil temperature at a depth of 12 cm was approximately 1.5 h and 2.5 h for minimum and maximum temperature, respectively.

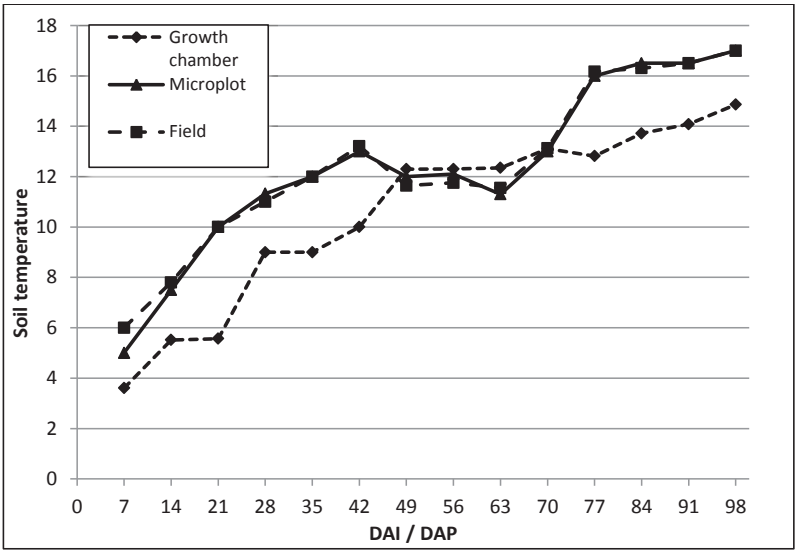


Figure 4.1. Soil temperatures (average day and night temperatures) in the growth chamber, microplots and fields (year 2013) at increasing days after infestation (DAI) or planting (DAP).

For each combination of PCN species and cultivar, four randomly selected plants were harvested weekly up to 14 weeks after inoculation. At each harvest, the whole plant was carefully removed from the pots. The roots were washed, blotted dry, weighed and cut into 1-2 cm pieces. A subsample of 5 g root was taken and stained with acid fuchsin (Byrd *et al.*, 1983). Developmental stages of PCN were determined and counted. At each sampling, a 200 cm^3 soil sample was taken from each pot and subjected to nematode extraction with automated zonal centrifugation (Hendrickx, 1995). The numbers of J2 and males were counted. When males and females were detected, an extra 1000 cm^3 soil sample was taken from each pot and cysts were extracted (Seinhorst, 1964). These cysts were counted before they were crushed and the number

of encysted eggs and J2 was determined. This was done for four replicates of each combination of nematode population and cultivar.

The number of accumulated DD required by the nematode to complete its life cycle (J2 to J2 of next generation) was calculated using the base temperatures of 6°C and 4°C for *G. rostochiensis* and *G. pallida*, respectively, according to Mugniéry (1978) and Langeslag *et al.* (1982). The modified average method was used to calculate the number of accumulated DD because at some observations the minimum temperature dropped below the base temperature; in these cases the base temperature was substituted for the minimum temperature. The more common average method can underestimate the number of degree days when the daily temperature fluctuates above and below the base temperature (Herms, 2004).

4.2.3 Microplot experiment

To monitor the life cycle of both *Globodera* species in natural conditions, a study was conducted outside in two microplots. In this way, nematode development was studied in the prevailing climatic conditions of 2013 and more space was available for root growth than in a pot. Also, no cysts from previous potato crops were present in the soil, allowing observation of only newly developed cysts. The microplots were laid out at the Institute for Agricultural and Fisheries Research (ILVO). Aluminium drums (80 cm diam., 55 cm deep) were filled with non-sterilised PCN-free loamy sand and planted with 18 sprouted potato tubers of cv. Eersteling or cv. Première on April 8 (Table 4.1). The same day, 570 cysts of *G. rostochiensis* (Kruishoutem) or 597 cysts of *G. pallida* (GOV 038) were placed in 30 retrievable nylon bags and buried in the microplot soil at a depth of 10 cm. For both nematode species, the initial population density was 2.7 eggs $(cm^3\ soil)^{-1}$. Plants were watered when needed and the soil temperature was recorded hourly throughout the whole experiment at a depth of 12 cm using a data logger.

Non-destructive root samples were taken from the 18 plants in each drum at different intervals (Table 4.2). Roots were washed free of soil and three 5 g subsamples were stained (Byrd *et al.*, 1983) to determine the nematode developmental stages. When present in the roots, J2 and males were also determined in a 200 cm^3 soil sample. If males and females were detected in the soil or roots, three 1000 cm^3 soil samples were taken to extract the cysts (Seinhorst, 1964).

These cysts were counted and their content was determined. Root and soil samples were taken daily, starting from 3 days before the set ultimate harvest date (June 20).

4.2.4 Field observations

The life cycle of PCN was also monitored in 2013 (*i.e.*, the same year as the microplot experiment) in potato crops in two fields (Tielt and Roeselare) with the aim of determining the date when the first cysts are formed in field situations. Both fields (sandy loam soil) were naturally infested with *G. pallida*. To determine spots with high incidence of PCN, fields were divided into plots of *ca* 20 m × 17 m containing eight rows of plants. One soil sample composed of 30 cores was taken 15 cm deep in each plot and the cyst population densities in the plots were estimated based on a subsample of 1000 cm^3 soil. For each field, a plot with a high density was selected for further observation: a plot with 22 eggs $(cm^3\ soil)^{-1}$ in Tielt and a plot with 15 eggs $(cm^3\ soil)^{-1}$ in Roeselare. In Tielt and Roeselare, the farmers decided to plant the early potato cvs Lady Christl (resistant to Ro1) and Sinora (resistant to Ro1), respectively, on 5 April (Table 4.1). In Roeselare, a granular nematicide (10 kg ha^{-1} Vydate, 10% (w/w) oxamyl) was applied in rows at planting.

Plants were grown according to common practice. Soil temperature was recorded hourly using a data logger with a sensor at 12 cm deep during the observations. In both selected plots, a sample was randomly taken from eight plants at different time points before and after the set ultimate harvest date (June 20) (Table 4.2).

The plants were not uprooted to avoid disturbing the crop; only a part of the root system of each plant was sampled together with surrounding soil, resulting in a sample of about 200 cm^3 soil and 5 g roots. At all sampling dates, the developmental stages of PCN were extracted and determined in a subsample of 100 cm^3 soil and 5 g root following the protocol mentioned above. Only cysts adhering to roots were counted as it was certain that they were new cysts. The numbers of cysts and their content were also determined in a soil sample taken on 20 June in each plot (20 m × 17 m), in the same way as before potato planting (30 cores, 15 cm deep, 1000 cm^3 subsample) to check if the *Globodera* population had increased during the short cultivation.

Table 4.2. Relationships between the occurrence of developmental stages of *Globodera* spp. and degree day accumulations on potato cultivars, in the fields and microplots.

	Microplot trial				**Field observations**	
Potato cultivar	Première		Eersteling		Sinora and Lady Christl	
Species	*G. pallida* (GOV 038)		*G. rostochiensis*		*G. pallida*	
Developmental stages	DAI	DD_4	DAI	DD_6	DAP	DD_4
J2 (soil)	21	87	24	71.3	27	101
J2 (root)	24	101	35	145	27	101
J3	35	296	35	145	38	296
J4	49	322	49	216	52	372
Male	59	391	59	291	62	421
Female	59	391	59	291	62	421
Cyst	65	463	68	401	68	463

DAI = days after infestation, DAP = days after planting, DD_4 = accumulated DD above 4°C, DD_6 = accumulated DD above 6°C. J2, J3, J4 = second-stage, third-stage, and fourth-stage juveniles, respectively.

4.2.5 Data analysis

For the growth chamber experiment, data on the final populations of *G. pallida* GOV 038 on three different cultivars and those of *G. pallida* Chavornay and GOV 038 populations on cv. Eersteling were statistically analysed using ANOVA. The differences among the final populations of *G. pallida* GOV 038 (number of eggs and juveniles) on three cultivars were compared with Tukey's Honestly Significant Difference (HSD) tests. The same test was performed to compare the final populations of eggs and juveniles of both *G. pallida* Chavornay and GOV 038 on cv. Eersteling. Data were considered significantly different when $P < 0.05$. All the analyses were performed with STATISTICA_9 software (Statsoft, OK, USA). No statistical analysis was performed on the field and microplot data because there were no replications of exact combinations of cultivars and populations.

4.3 Results

4.3.1 Life cycle of *G. pallida* and G. *rostochiensis* in growth chamber experiment

For all three cultivars, hatched J2 of *G. pallida* GOV 038 were first found in the soil 28 days after infestation (DAI); at that time, the temperature was set at 8.8°C and the roots had started to develop. The accumulated heat to reach this stage was 84 DD_4 (Figure 4.2). Second-stage juveniles of *G. pallida* GOV 038 continued to be present in the soil until 84 DAI, the highest numbers being observed at 35 and 42 DAI (Figure 4.3). Thereafter, J2 in the soil decreased in number.

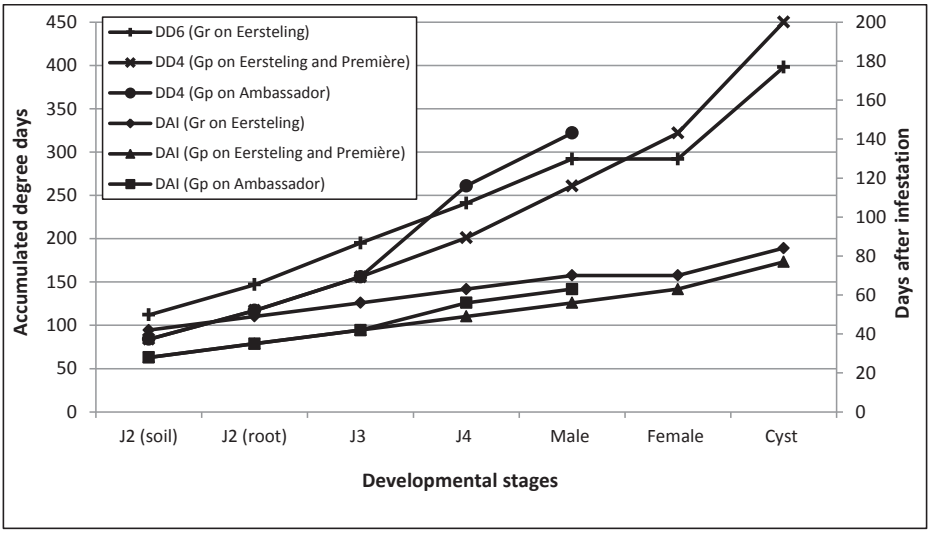


Figure 4.2. Growth chamber experiment. Occurrence of developmental stages of *Globodera* spp. (populations) and heat accumulation (DD) on potato cvs Eersteling, Première and Ambassador (temperature following 6-years statistics in growing period of early potatoes). On cv. Ambassador, no females or cysts of *G. pallida* were formed. DAI = days after infestation, DD_4 = accumulated DD above 4°C, DD_6 = accumulated DD above 6°C, Gr = *G. rostochiensis*, Gp = *G. pallida*.

In the roots, the first J2 of *G. pallida* GOV 038 were detected on all three cultivars at 35 DAI; the temperature in the growth chamber was then set at 9.1°C and the accumulated heat was 117 DD_4 (Figure 4.2). Third-stage juveniles (J3) were first found in roots of the three cultivars at 42 DAI. On cvs Eersteling and Première, fourth-stage juveniles (J4), males and females of *G. pallida* GOV 038 were extracted from the roots at 49, 56 and 63 DAI, respectively (Figures 4.2,

4.4). When the first females appeared, the accumulated heat was 322 DD_4 (Figure 4.2). Newly developed tubers were observed at 49 DAI. The first cysts of *G. pallida* GOV 038 were found on the roots of cvs Eersteling and Première at 450 DD_4, *i.e.*, 77 DAI (Figure 4.2). On the tubers, white females and cysts were found at 519 DD_4, *i.e.*, 84 DAI. Development of *G. pallida* GOV 038 was delayed on cv. Ambassador; J4 and males were observed at 56 and 63 DAI, respectively, *i.e.*, 1 week later than on cvs Eersteling and Première. Females were not observed on this partially resistant cultivar during the whole observation period (Figures 4.2-4.4). Life stages of *G. pallida* GOV 038 occurred similarly on cvs Eersteling and Première and also population increase was similar: no significant difference was found for the final number of cysts on cv. Eersteling (*Pf*/*Pi* = 9.2) and on Première (*Pf*/*Pi* = 8.9) ($F = 0.62$; $df = 1$; $P = 0.46$) or for the final number of eggs and juveniles on both cultivars ($F = 0.58$; $df = 1$; $P = 0.81$) (Table 4.3; Figure 4.5).

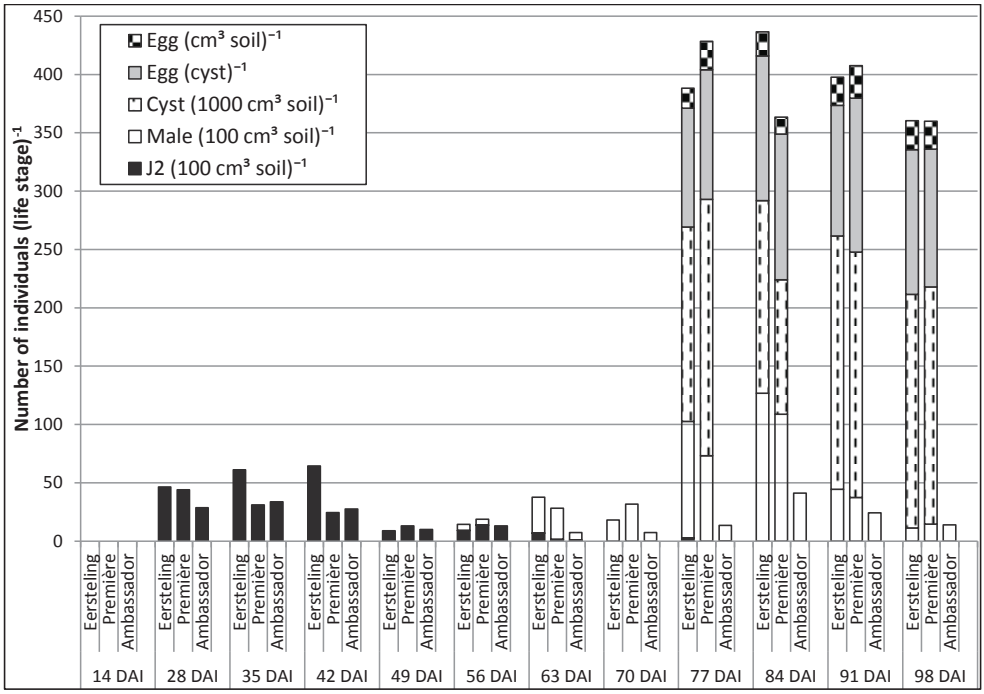


Figure 4.3. Growth chamber experiment. Quantification of life stages of *Globodera pallida* GOV 038 in soil planted to different potato cultivars at increasing days after infestation (DAI).

The results obtained for cv. Eersteling inoculated with *G. pallida* Chavornay were similar to those acquired with *G. pallida* GOV 038 (Figures 4.2, 4.6, 4.7). However, the number of cysts ($F = 4.35$; $df = 1$; $P < 0.05$), and the number of newly formed eggs and juveniles of *G. pallida* Chavornay, were significantly higher than those of *G. pallida* GOV 038 on cv. Eersteling at the final observation 98 DAI ($F = 5.45$; $df = 1$; $P < 0.05$) (Table 4.3; Figure 4.5). The multiplication rate based on numbers of eggs (*Pf*/*Pi*) was 12.0 for *G. pallida* Chavornay, whereas *Pf*/*Pi* was 9.2 for *G. pallida* GOV 038. The early stages of the life cycle of *G. rostochiensis* in pots with cv. Eersteling appeared later and needed more heat units than those of both *G. pallida* populations.

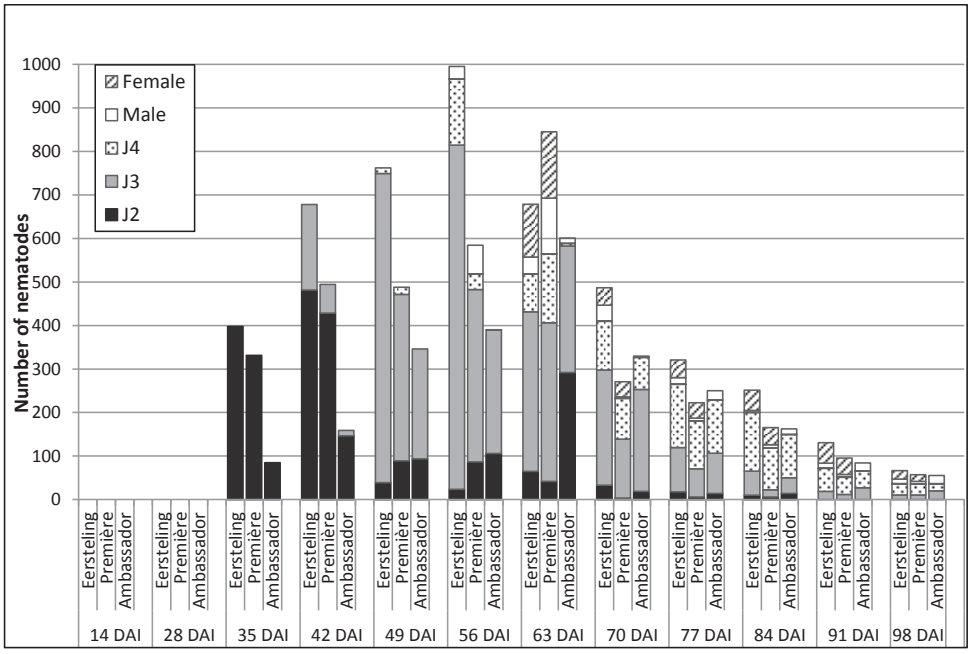


Figure 4.4. Growth chamber experiment. Quantification of life stages of *Globodera pallida* GOV 038 in 5 g root of different potato cultivars at increasing days after infestation (DAI).

Second-stage juveniles of *G. rostochiensis* were detected in soil 42 DAI when the temperature was set at 10.1°C and 112 DD_6 were accumulated (Figure 4.2). Second-stage juveniles were detected in the roots of cv. Eersteling 49 DAI (12.3°C) at 147 DD_6. Third-stage juveniles and J4 were found in the roots 56 and 63 DAI, respectively. Eventually, males, females and cysts of *G. rostochiensis* were found 70, 70 and 84 DAI, respectively, corresponding with

292, 292 and 398 DD_6 (Figures 4.2, 4.6, 4.7). Fewer heat units were needed for the development of females and cysts of *G. rostochiensis* than for development of females and cyst of both populations of *G. pallida*.

Table 4.3. The final population densities of *Globodera* spp. (*P*f) and the multiplication rates (*Pf*/*Pi*) observed on potato cultivars under growth chamber conditions (*P*i = 2.7 eggs (cm^3 soil)$^{-1}$).

	***G. pallida* GOV 038**		***G. pallida* Chavornay**		***G. rostochiensis***	
Potato cultivar	***Pf* (eggs (cm^3 soil)$^{-1}$)**	***Pf*/*Pi***	***Pf* (eggs (cm^3 soil)$^{-1}$)**	***Pf*/*Pi***	***Pf* (eggs (cm^3 soil)$^{-1}$)**	***Pf*/*Pi***
Eersteling	25 a*	9.2	32.4 *	12	7.8	2.9
Première	24.1 a	8.9	-	-	-	-
Ambassador	0 b	0	-	-	-	-

Data were analysed with one-way ANOVA and different letters indicate significant differences ($P \leq 0.05$) among three cultivars. An asterisk indicates differences between the final population densities of *G. pallida* GOV 038 and Chavornay, obtained through Tukey's Honest Significant Difference (HSD) tests.

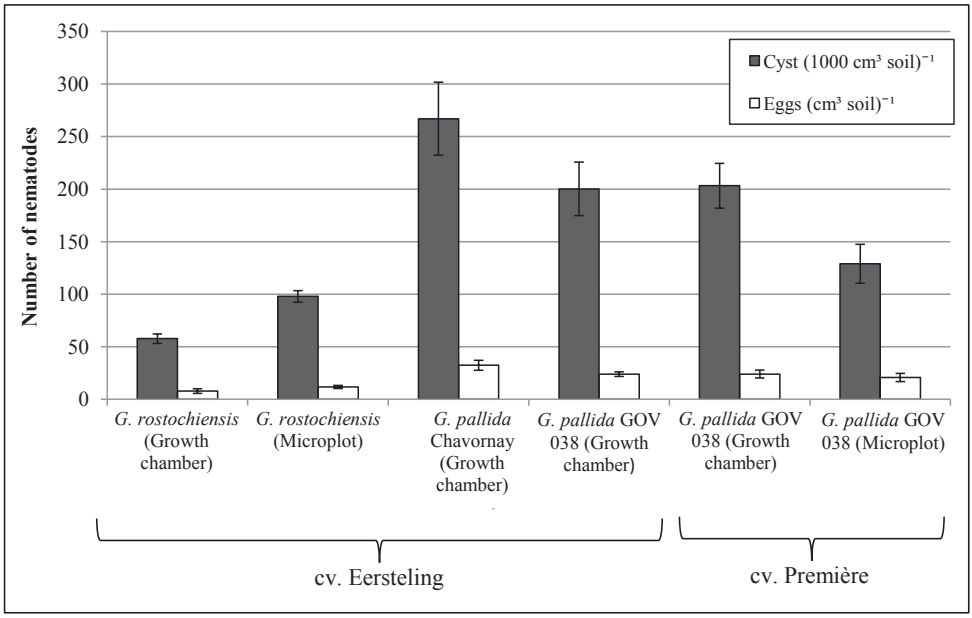


Figure 4.5. Final population densities of *Globodera pallida* and *G. rostochiensis* in the growth chamber and microplots after infestation with cysts at *P*i= 2.7 eggs (cm^3 soil)$^{-1}$ on June 20 (ultimate harvest date).

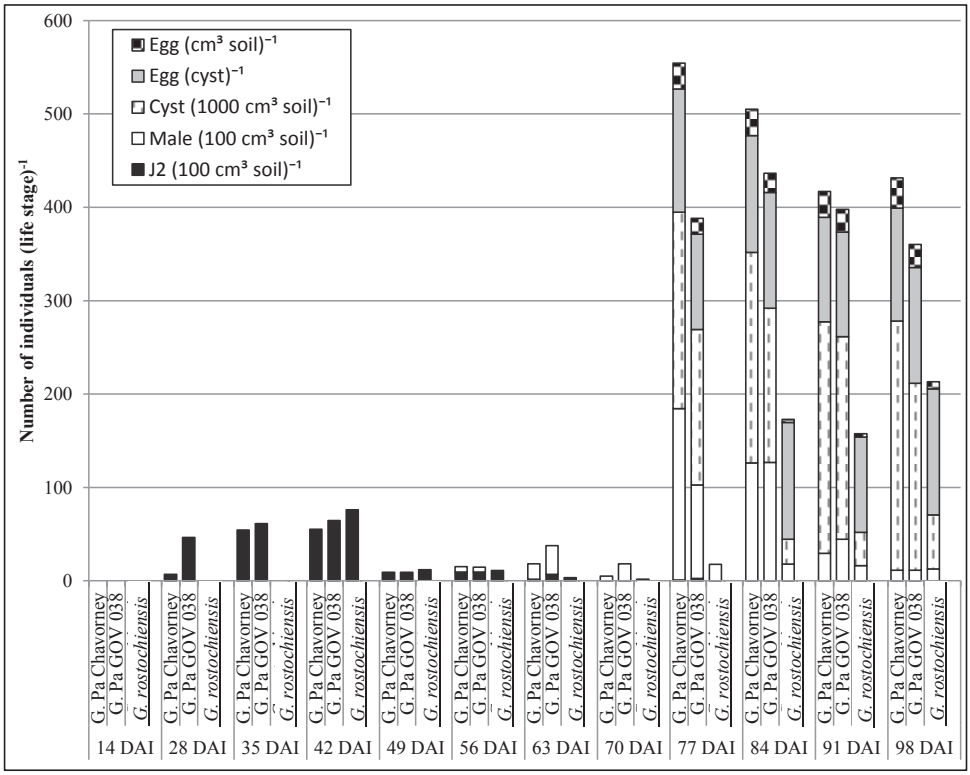


Figure 4.6. Growth chamber experiment. Quantification of life stages of *Globodera pallida* Chavornay, *G. pallida* GOV 038 and *G. rostochiensis* in soil planted to potato cv. Eersteling at increasing days after infestation (DAI). G. Pa = *G. pallida*.

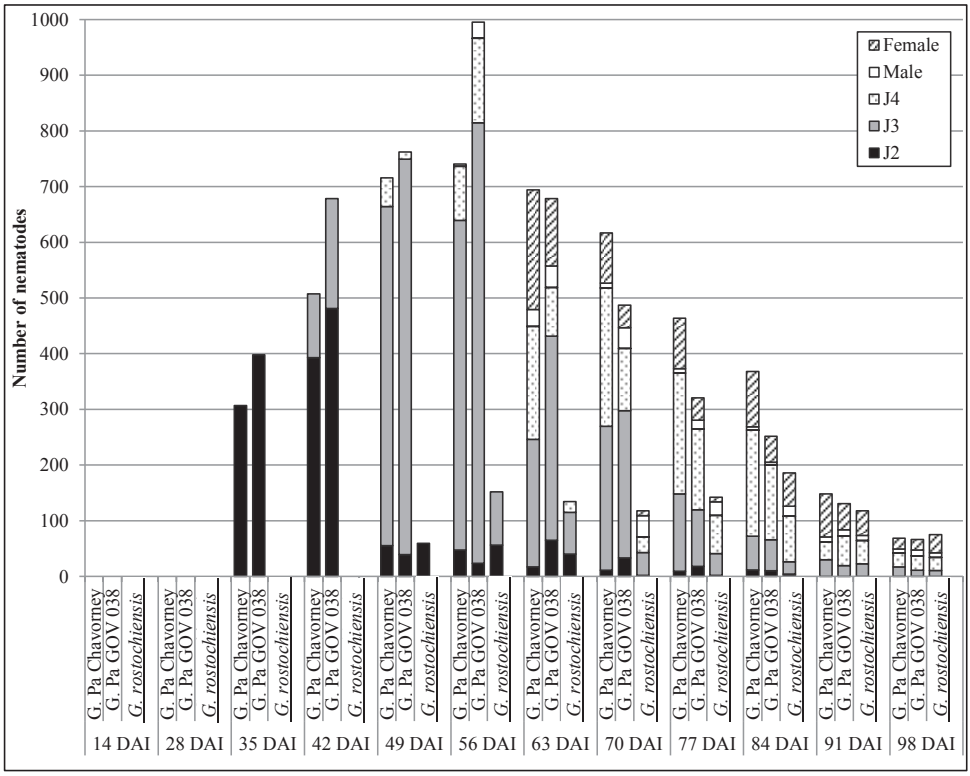


Figure 4.7. Growth chamber experiment. Quantification of life stages of *Globodera pallida* Chavornay, *G. pallida* GOV 038 and *G. rostochiensis* in 5 g root of potato cv. Eersteling at increasing days after infestation (DAI). G. Pa = *G. pallida*.

4.3.2 Microplot experiment

In the microplot planted to cv. Première, the first J2 of *G. pallida* GOV 038 were detected in the soil 21 DAI, when the soil temperature was 10°C and the accumulated DD were 87 DD_4 (Table 4.2). In the roots, J2 were already observed 24 DAI (soil temperature = 11°C). Later stages, *i.e.*, J3, J4, males and females, were found for the first time in the roots at 35, 49, 59 and 59 DAI, respectively. The accumulated DD to reach the male and female stages were 391 DD_4. The first cysts were observed on the roots on June 12 (=65 DAI) at 463 DD_4, *i.e.*, about 1 week before the harvest date (=June 20) (Table 4.2; Figures 4.8, 4.9). On June 20, 129 cysts were extracted from 1000 cm^3 soil. The average cyst content was 162 $\pm$ 26 eggs and J2.

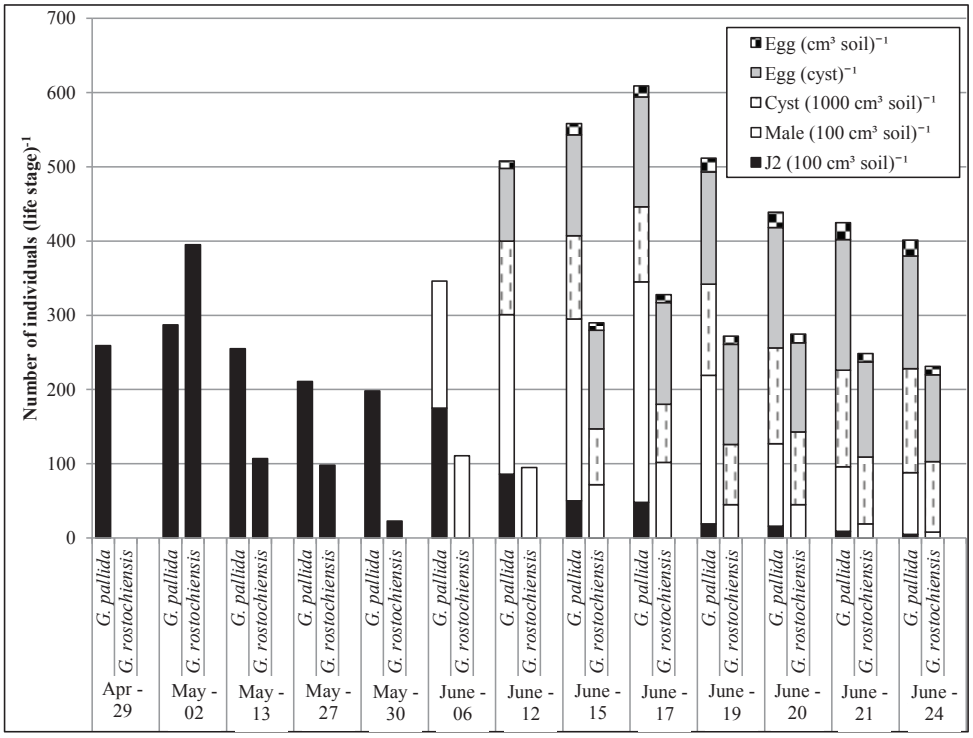


Figure 4.8. Microplot experiment. Quantification of life stages of *Globodera pallida* GOV 038 and *G. rostochiensis* in soil at increasing time after infestation.

In the soil of the microplot planted to cv. Eersteling, J2 of *G. rostochiensis* were detected 24 DAI when the soil temperature was 11°C. In the roots, J2, J3 and J4 were observed 35, 35 and 49 DAI, respectively. As observed for *G. pallida*, both males and females were found in the roots 59 DAI (DD_6 = 291). However, cysts of *G. rostochiensis* were observed on June 15, *i.e.*, 3 days later than those of *G. pallida* corresponding to 401 DD_6 (accumulated heat above 6°C) (Table 4.2; Figures 4.8, 4.9). On June 20, 98 cysts were detected in 1000 cm^3 soil. The average number of eggs and juveniles per cyst was 120 ± 15.

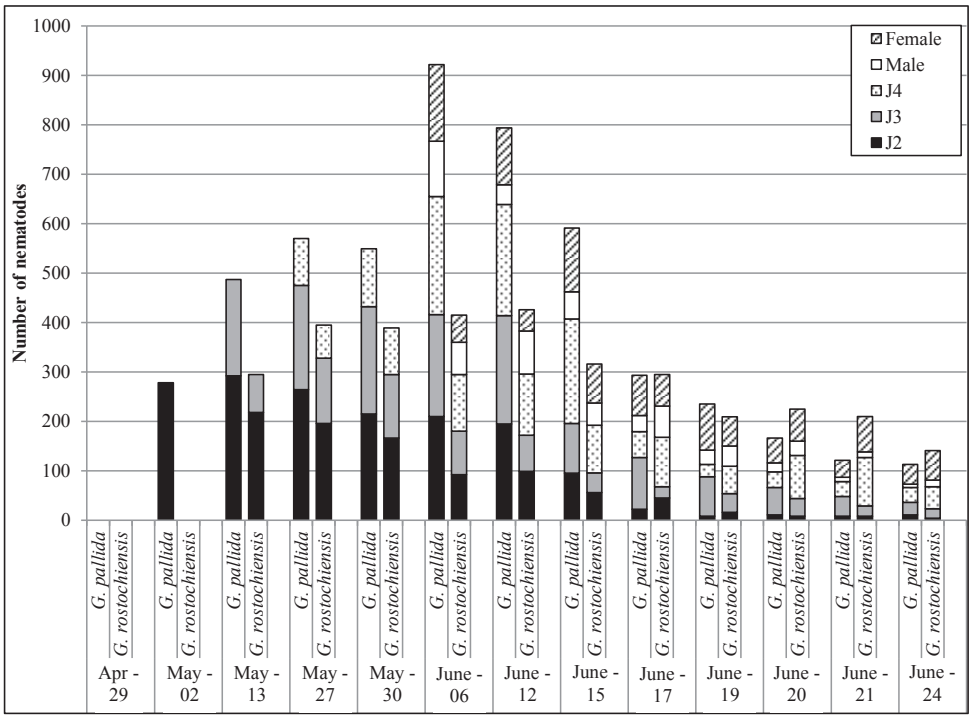


Figure 4.9. Microplot experiment. Quantification of life stages of *Globodera pallida* GOV 038 and *G. rostochiensis* in 5 g root at increasing time after infestation.

4.3.3 Field experiment

In the field in Roeselare, *G. pallida* completed its life cycle on cv. Sinora before the set harvest date (June 20). Second-stage juveniles were first observed in the roots 27 days after planting (DAP) (soil temperature = 11°C, DD_4 = 101). Third-stage juveniles and J4 were found in the root 38 and 52 DAP, respectively. Both males and females were detected 62 DAP at 421 DD_4. 26 cysts attached to 5 g roots in 100 cm^3 soil were observed on June 12 (=70 DAP, 463 DD) (Table 4.2; Figures 4.10, 4.11). On June 20, 144 cysts were detected on 5 g roots in 100 cm^3 soil containing 193 $\pm$ 25 eggs and juveniles per cyst.

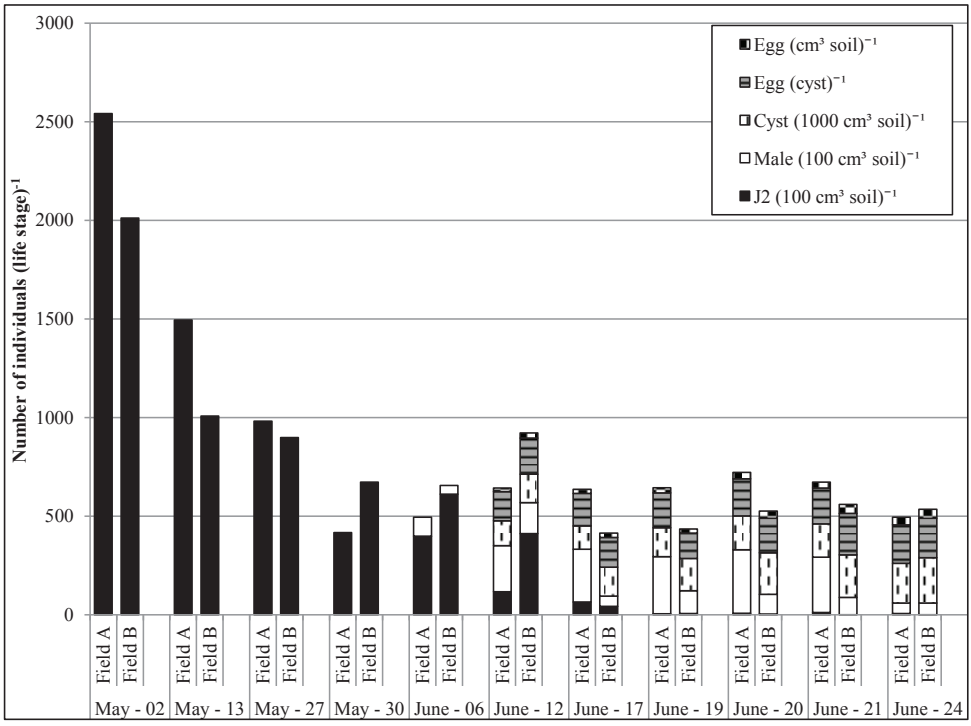


Figure 4.10. Field observation. Quantification of life stages of *Globodera pallida* in soil at increasing time after planting in the field (A: Roeselare, B: Tielt).

The occurrence of all developmental stages of *G. pallida* on cv. Lady Christl in the field in Tielt was similar to that in Roeselare. The first cysts also were observed on June 12: 72 cysts were found on 5 g roots (Figure 4.11). On June 20, 198 cysts were detected on 5 g roots, with 175 ± 51 eggs and J2 per cyst.

In both locations, the population densities of *G. pallida* had increased by June 20: multiplication rates (*Pf*/*Pi* based on eggs and juveniles) of 2.2 and 1.6 were determined for the plots in Roeselare and Tielt, respectively.

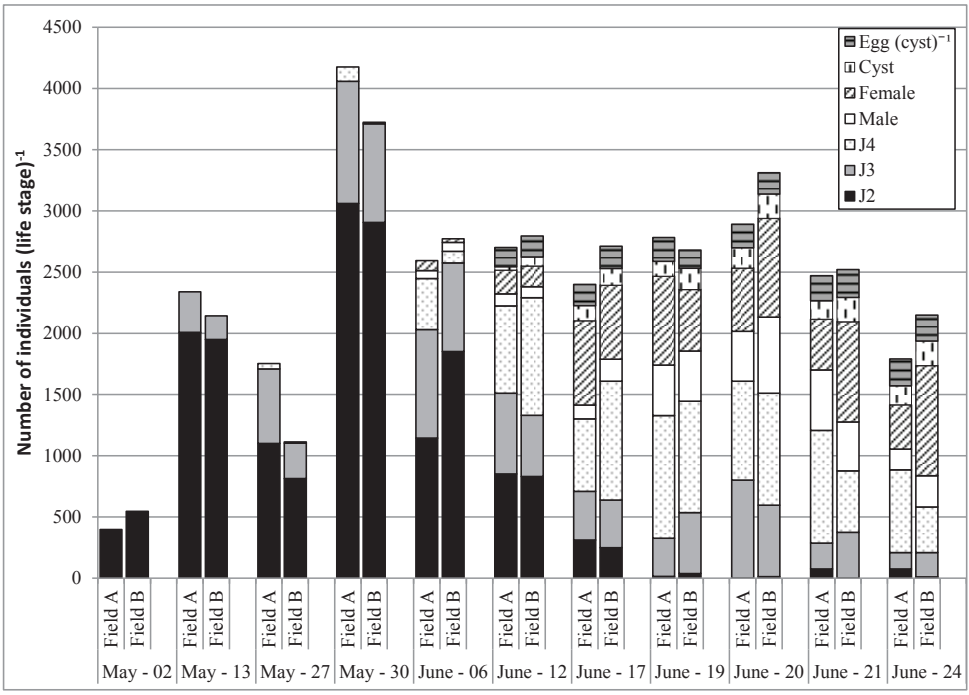


Figure 4.11. Field observation. Quantification of life stages of *Globodera pallida* in 5 g root at increasing time after planting in the field (A: Roeselare, B: Tielt).

4.4 Discussion

This study confirms the impact of soil temperature, cultivar and nematode species/population on the different phases in the life cycle of PCN on potato. It is known that soil temperature greatly influences hatching, mobility, infectivity and lipid utilisation of J2 of PCN (Robinson *et al.*, 1987a).

In both the growth chamber experiment and the microplot, J2 of *G. pallida* hatched when soil temperature had reached 8.8-10°C. However, this temperature window was too low for J2 of *G. rostochiensis* to hatch; J2 of this species hatched only when the soil temperature reached 10.1-11°C. In Italy, Greco *et al.* (1988) reported 10°C as minimum temperature for J2 of *G. rostochiensis* to hatch, whereas in Finland the first hatched J2 of *G. rostochiensis* were already observed at 4-5°C (Tiilikala, 1987). Kaczmarek *et al*. (2015) also demonstrated that low soil temperatures are likely to favour *G. pallida,* whereas warmer temperatures are likely to favour

G. rostochiensis. Apparently, temperature requirements for hatching not only differ between PCN species, they also differ between populations of the same species. Ellenby & Smith (1975) found a difference in response to low temperature (10°C) between two populations of *G. rostochiensis* in Newcastle (northeast England) and Ayrshire (south west Scotland). The latter population showed adaptation to its local cultural conditions and had a high hatching rate (3260 J2 from 100 cysts) at 10°C; at the same temperature the population from Newcastle produced only 148 J2 from 100 cysts. Franco (1979) revealed that a Peruvian population of *G. pallida* was less sensitive to temperature extremes of 5°C and 30°C than two British populations of the same species. The results of the growth chamber experiment showed that the J2 of both *G. pallida* populations hatched at 8.8°C; however, *G. pallida* hatched at a higher temperature (10°C) in the microplot.

It is remarkable that although the estimated base temperature for *G. rostochiensis* and *G. pallida* is 5.9 and 3.9°C, respectively (Mugniéry, 1978), in these trials J2 of both species hatched only at higher temperatures, *i.e.*, 10.1-11°C for *G. rostochiensis* and 8.8-10°C for *G. pallida*. This might be attributed to the fact that next to their apparent need of a soil temperature above the basal value, both PCN species are highly dependent on host root diffusate for hatching (Perry, 1997). In Belgian early potato-growing regions, soil temperature is low during the early period of the crop and rises slowly during the first weeks after planting. This results in a slow root development, as observed in the growth chamber experiment with simulated early season temperatures. The meagre root development most probably results in poor release of root diffusate and low concentrations of the hatching factors, reducing the hatching rate of PCN juveniles. Rawsthorne & Brodie (1986) demonstrated a positive relationship between the root weight and the hatching rate during the first 3 weeks. Turner & Stone (1981) also reported that larger root systems may have higher concentrations of hatching factors.

On the other hand, the impact of other environmental factors on the hatching process, such as soil moisture, soil microorganisms, soil minerals and organic substances should also be considered. Devine & Jones (2000) found that different soil types may affect the availability and movement of hatching factors in the soil as hatch of *G. pallida* was increased in the highly organic soil and in a sandy soil.

Differences in temperature requirements between PCN species were also observed for the further stages in the development. In this study, *G. rostochiensis* developed slower than *G. pallida*; females of the former species appeared later than those of *G. pallida*. Foot (1978) and Webley & Jones (1981) reported a similar behaviour of both species. Monitoring the life cycle of PCN in the growth chamber and the microplot showed that at low temperatures, *G. pallida* secures its advantage over *G. rostochiensis* with faster hatching. However, although females and cysts of *G. rostochiensis* appeared later than those of *G. pallida*, fewer accumulated heat units were required by *G. rostochiensis* for female and cyst development, due to its higher base temperature. There was no difference in the speed of development between two populations of *G. pallida* on cvs Eersteling and Première. They both completed their life cycle at 450 DD_4. However, the development of *G. pallida* GOV 038 was delayed and never completed on cv. Ambassador (partially resistant to Pa2 and Pa3).

In the growth chamber experiment, J2 of both populations of *G. pallida* (Chavornay and GOV 038) penetrated the tubers of cvs Eersteling and Première and white females and cysts were found at 519 DD_4, *i.e.*, 84 DAI. These cysts were from the first generation because no second peaks of J2 were observed in the soil after the formation of the cysts on the roots.

In the growth chamber where the temperature was set according to the averages of 6 years of temperature records, cysts of *G. pallida* and *G. rostochiensis* were observed at 450 DD_4 and 398 DD_6, respectively. These values were close to those registered for both species in the microplots and fields (463 DD_4 and 401 DD_6 for *G. pallida* and *G. rostochiensis*, respectively). In Italy, *G. rostochiensis* required 126 and 168 DD in subtropical areas and 275 and 450 DD in temperate regions to reach the female and cyst stages, respectively (Greco *et al.*, 1988). The latter values (temperate regions) are close to the accumulated DD required by this species in my microplot (291-401 DD) and pot study (292-398 DD). In Cyprus, *G. rostochiensis* completed its life cycle on autumn and spring crops at 56 and 63 days after planting, respectively, when the accumulated DD were 416 and 529 (Philis, 1980). In Spain, *G. pallida* completed its life cycle on the early potato cv. Maris Peer at 540 DD, while on the late potato cv. Marfona, planted mid-January and harvested 17 May, females were observed at 400 DD (Alonso *et al.*, 2011) close to those observed in the present study (391-421 DD_4). Fluctuating and lower soil temperatures during the growing period of early potatoes (November-April) in Spain may account for the

larger amount of accumulated heat required. Apparently, the number of DD required by PCN varies between locations and between early or late plantings. This might be due to the difference between the base temperatures of *Globodera* spp. However, it should be noted that different accumulated DD estimates reported for PCN species might be partially due to the method of calculating accumulated heat. Therefore, it is strongly recommend that authors describe precisely the method of computing the number of DD, so that the results can be interpreted and compared by others.

In the field and in the microplots, both *Globodera* species needed about the same amount of accumulated heat for cyst development. This confirms that microplot studies offer a good alternative to field observations with the advantage of harvesting only new cysts (if the initially inoculated cysts are confined, *e.g.*, in small bags). Most studies in growth chambers to investigating nematode population dynamics set the temperature at constant levels during the whole experiment (*e.g.*, Foot, 1978; Franco, 1979; Blok *et al.*, 2010). However, my study tried to simulate the real external (field) conditions by changing day and night temperatures and increasing temperatures weekly, similar to growth conditions in the field.

As could be expected, the occurrence of different life stages of PCN varied between the cultivars. The growth chamber experiment showed that the development of J3, J4 and adults of *G. pallida* GOV 038 was significantly delayed on cv. Ambassador, a cultivar partially resistant to *G. pallida*. On this cultivar, J4 were observed 2 weeks later than on the susceptible cvs Eersteling and Première. During the course of the experiment, females were never observed in the roots of cv. Ambassador. This absence should be explained by the resistance mechanism; *i.e.*, a sequence of stimulation of hatching and invasion, followed by the egression of J2 or improper establishment of feeding sites (Mullin & Brodie, 1988). No egression of J2 was observed as numbers of J2 in soil were declining over time. However, males were detected in both soil and roots of cv. Ambassador; this could be supporting evidence of the effect of resistance on sex reversal of juveniles, which could be attributed to improper establishment of feeding sites. Indeed, two developing ovaries were observed in some J4, typical for female J4, but females were never found. Heijbroek (1977) suggested a possible influence of partial resistance on the sex ratio of beet cyst nematodes. Some inhibitors also influenced the sexual fate of J2 of *G. pallida* in transgenic potatoes (Atkinson *et al.*, 2003).

In the growth chamber experiment, the developmental stages of *G. pallida* GOV 038 were observed simultaneously on cvs Eersteling and Première. There was no significant difference between final population densities of *G. pallida* GOV 038 on these cultivars. The high final population of Chavornay confirmed its high multiplication capacity. This virulent population did not hatch earlier nor did it develop any faster than the local population GOV 038. The occurrence of *G. pallida* stages on cvs Sinora and Lady Christl in the fields was similar.

In Belgium, it is assumed that harvesting early potato cultivars before June 20 would interrupt the life cycle of PCN (Anon., 2010). However, the results obtained in the growth chamber as well as in the microplots and fields demonstrate that females and cysts of populations of *G. pallida* and *G. rostochiensis* can appear before the mentioned ultimate harvest date. In the microplot, J2 of *G. rostochiensis* hatched 24 days after cyst inoculation and the life cycle was completed 68 DAI. The heat required was similar to that in the growth chamber (401 and 398 DD). The soil temperature was similar in the fields and in the microplot, however, it was lower in the growth chamber. This could explain the earlier emergence of J2 and the shorter duration of the life cycle of *G. pallida* in the fields and in the microplot than in the growth chamber. Therefore, the differences in time (hence, date) needed for development of cysts is dependent on the prevailing temperatures and can differ from year to year (here growth chamber *vs* real time temperatures in microplot and field). Nevertheless, new cysts had always formed before June 20. Moreover, due to the cold weather conditions in the early spring of 2013, 'early' potato was planted at the beginning of April, whereas this is usually done in March, or even middle to late February (sometimes under plastic shade). The extreme 'late' conditions of the microplot and field trials in 2013 emphasise that cyst development is still possible before the set harvest date of 20 June.

Next to the role of the prevailing temperature, other biotic and abiotic factors, *e.g.*, soil moisture, soil aeration, soil structure or soil microorganisms, might have made the environment more suitable to emergence of J2 than under controlled conditions and in a sterilised soil (Robinson *et al.*, 1987a, b; Ryan & Jones, 2003). In this study, sterilised soil was used in the growth chamber experiment. Previous studies showed that the presence of some microorganisms in the soil or their metabolites might stimulate or inhibit hatching (Deliopoulos *et al*., 2007; Lettice & Jones, 2015). Hence, hatching factor-producing microorganisms in non-sterile soil in

the microplots and in the fields might have caused the earlier PCN hatch than in the growth chamber.

Despite the early planting, the PCN populations were able to increase in all experiments, except on the resistant variety. In the field, the multiplication rate of *G. pallida* was greatly inferior (*Pf*/*Pi* = 1.6-2.2) to the rate observed in both the microplot (*Pf*/*Pi* = 9.5) and pot trials (*Pf*/*Pi* = 8.9-12). This difference can reflect the density dependent effect of increased competition for feeding sites and food reserves at high initial population densities (*Pi*) in the fields (15 and 22 eggs (cm^3 soil)$^{-1}$) compared to the low *Pi* (2.7 eggs (cm^3 soil)$^{-1}$) in the artificially infested microplots and pots (Seinhorst, 1967; LaMondia & Brodie, 1986; Phillips *et al.*, 1991). Further field observation is required to monitor the population build-up of PCN at different *Pi*.

Notwithstanding the application of the granular nematicides oxamyl in the field in Roeselare, the pattern of the PCN development was similar to that in the untreated field in Tielt. However, Evans & Wright (1982) showed that oxamyl affected the host-finding ability of J2 of *G. rostochiensis* and delayed the development of juveniles in the roots. Molendijk *et al.* (2010) also reported that the full field application of 40 kg oxamyl $(ha)^{-1}$ in fields naturally infested with *G. pallida* caused a reduction of 38% of the maximum number of cysts. By contrast, in my study, the field observation revealed that oxamyl did not reduce the number of cysts and eggs + J2 per cyst and cm^3 soil at harvest possibly due to the lower application rate of oxamyl. These findings are in agreement with those of Whitehead *et al.* (1984). This might also be attributed to the long hatching period of *G. pallida* compared with that of *G. rostochiensis* contributing to its presence in the soil over a long period, extending the time when most of the active substance of oxamyl has been degraded. It was shown that after incorporation, the concentration of granular nematicides, *e.g.*, oxamyl, will decrease due to degradation of the nematicide, which could be influenced by the environment (*e.g.*, temperature), soil properties and microbial activities in the soil (Smelt *et al.*, 1987; Haydock *et al.*, 2012). This chemical could persist in the soil for 28-91 days after application (Haydock *et al.*, 2012).

To conclude, these observations showed that both *Globodera* species are able to mature on different cultivars at low temperatures prevailing in spring before the harvest date of 20 June and allow the increase of the PCN population in the fields. Kaczmarek *et al*. (2015) suggested

that increases in soil temperatures due to regional climatic differences or climate change are likely to favour PCN multiplication because they observed the hatching response for both PCN species was greater and faster at the higher temperatures. Ambassador, a partially resistant cultivar to *G. pallida*, did not allow any multiplication of this PCN species in the growth chamber conditions. This indicates the efficacy of this cultivar as a trap crop. Hence, the precise identification of the PCN species and the pathotype present in the field is needed to choose the appropriate potato cultivars that can influence the effectiveness of planting early potato cultivars in trap cropping and reduce the population build-up of PCN. However, the coexistence of *G. rostochiensis* and *G. pallida* in the fields should be considered because high-yielding early potato cultivars with significant levels of resistance to either *G. pallida* or to both PCN species are only available in limited numbers. This research determined the accumulated DD required by PCN species to develop different life stages in natural fluctuating soil temperatures. Therefore, harvesting based on the accumulated heat above the basal development temperature required by PCN species can replace the set harvest date of 20 June. However, it should be noted that based on the 6-year weather statistics of the growing season of early potato growing regions, farmers should have harvested their potato by 8, 10, 11 and 12 June, before the formation of the cysts. The average yield for potato harvested by these dates is approximately 10 tonnes per hectare lower than that harvested by June 20 resulting in about 7000 to 11000 euro loss per hectare (K. Demeulemeester, pers. comm.).

Chapter 5

Optimising trehalose-based quantification of live eggs in potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*)

Ebrahimi, N., Viaene, N. & Moens, M. (2015). Optimizing trehalose-based quantification of live eggs in potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*). *Plant Disease* 99, 947-953.

5.1 Introduction

Knowledge of the initial population densities and viability of potato cyst nematode eggs in fields is required for decision-making in an integrated management program. Seinhorst (1967) demonstrated that crop yield is strongly influenced by pre-plant (initial) nematode densities (Pi) which is estimated through soil sampling of the field, followed by extraction of the cysts from the sample, determination of the content of the cysts (sometimes based on a subsample), and expressed as the number of eggs and second-stage juveniles (in the cysts) per volume of soil. The damage threshold of PCN in potatoes is 1 to 2 eggs per g soil (Turner & Subbotin, 2013). If the cyst content is completely viable, the Pi is estimated correctly. However, cysts may contain non-viable eggs or J2. In that case, the Pi will be overestimated, leading to needless management efforts. Therefore, information on the viability of eggs and J2 in the cyst would make the management of PCN more accurate. The viability of encysted eggs is also a critical issue in the interpretation of results of the statutory pre-plant sampling for seed potato production as well as for certification of other kinds of plants for planting where no viable egg should be present (Anon., 2007). According to the EU legislation, if PCN are found in a field, no seed potato can be produced in this field; however, ware potatoes may be grown provided officially approved management practices are applied to suppress the PCN and minimize their spread.

The viability of the cyst content of PCN can be measured by a variety of methods, *viz.* visual assessment based on morphology, Meldola's blue staining followed by microscopic observations, hatching assays, or plant infectivity assays (OEPP/EPPO, 2013). However, the first three aforementioned methods contain an element of subjectivity or are labour intensive and time consuming. Van den Elsen *et al*. (2012) reported a qualitative viability test based on the detection of trehalose in live eggs. The assay, however, was not applied to enumerate live eggs. Recently, a quantitative PCR (qPCR)-based method in combination with a photoreactive DNA-intercalating dye (propidium monoazide, PMA) has been developed for the quantification of viable PCN eggs (Christoforou *et al*., 2014). Compared with this method, a quantitative technique based on trehalose seems to be a feasible and more affordable method.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a disaccharide present in the perivitelline fluid between the unhatched J2 and the eggshell. Eggs of *G. rostochiensis* contained

6.4% trehalose at a concentration of 0.34 M (Clarke & Hennessy, 1976; Perry, 1989). Because of the limited permeability of the lipid layer of the eggshell, the trehalose is retained within the egg. In the absence of a suitable host, PCN survive as J2 inside the eggs within the cyst. Under the influence of host root diffusate the permeability of the inner lipoprotein membranes of the eggshell increases, the trehalose diffuses out of the eggs and J2 hatch from the eggs (Clarke & Perry, 1977; Clarke *et al*., 1978). Changes in the permeability of the eggshell upon death of the J2 embedded in the egg, also lead to the diffusion of trehalose out of the eggs. As a consequence, the presence of trehalose in the egg is an indication of the viability of eggs. Assessing the viability of the egg content by estimating the trehalose content of the egg includes the hydrolysis of trehalose into two glucose molecules followed by the detection of glucose.

The goal of this research was to obtain an optimised method to quantify live eggs of PCN. I did this using a previously described trehalose-based method by van den Elsen *et al*. (2012) for the qualitative determination of viability of PCN. Specific questions which were addressed included *i*) development of a method to determine the number of viable eggs of PCN in a sample, using the quantity of trehalose present in the eggs, and assessment of its sensitivity *ii*) evaluation of the reliability of this trehalose-based method by comparing it with commonly used viability assays, and *iii*) assessment of the robustness of the quantitative trehalose-based method by using cysts of both PCN species.

5.2 Materials and methods

5.2.1 Potato cyst nematode cultures

Populations of *G. rostochiensis* (Kruishoutem, East Flanders, Belgium) and of *G. pallida* (Chavornay, Switzerland) were maintained on potato cv. Désirée under greenhouse conditions (see section 3.1). Cysts were kept for at least 4 months at 4°C to overcome diapause before use in experiments.

5.2.2 Determining viability of cultured cysts by hatching assays and visual assessment

The viability of the cultured eggs that were used in subsequent experiments was assessed with a hatching assay and visual assessment. For hatching assays, potato root diffusate (PRD) was collected from potato cv. Désirée following the protocol described by Turner *et al*. (2009)

(see section 3.3). Four replicates of 20 intact cysts of *G. rostochiensis* or *G. pallida* were soaked in water for 48 h before being exposed to PRD (see section 3.4). The samples were maintained at 21°C in a completely randomized design. The number of hatched J2 was counted at weekly intervals over a period of 14 weeks and the PRD was renewed at every counting event. After 14 weeks, cysts were crushed and the number of viable unhatched J2 inside the cysts was counted. This number, together with the number of hatched J2, allowed for the calculation of the percentage hatch of eggs. The same procedure was applied in further experiments where hatching in PRD was used as a reference test, but with different numbers of cysts and for varying periods of time.

For the visual determination of the viability of cultured eggs, five replicates of 20 cysts of *G. rostochiensis* and of *G. pallida* were soaked in tap water for 24 h at 21°C before releasing the eggs inside the cysts by crushing (see above). The volume of the egg suspension obtained from each replicate (20 cysts) was increased to 50 ml. The number of viable eggs in the suspension was determined based on three 3 ml subsamples using a microscope (100 ×). Damaged and empty eggs with a non-smooth eggshells, and shrivelled disintegrated J2 with no clear lip region or stylet, were considered dead as described in the EPPO protocol PM7/41(2) (OEPP/EPPO, 2013). The visual assessment of viability of eggs of both PCN species was performed twice.

5.2.3 Quantification of trehalose in cysts and construction of a standard curve

Trehalose in eggs of non-crushed cysts of *G. rostochiensis* or *G. pallida* was measured using a detection kit (K-TREH 01/09, Megazyme International Ireland Ltd., Wicklow, Ireland) following the protocol of van den Elsen *et al*. (2012). Single or multiple cysts of *G. rostochiensis* or *G. pallida* were transferred to 0.2 ml Eppendorf tubes containing 10 µl MilliQ water. Blank samples containing 10 µl MilliQ water without any cyst were also included in the experiment. Samples were incubated for 30 min at 99°C in a thermomixer (HLC, MHR 13, Pforzheim, Germany) at 300 rpm to extract trehalose. Samples were cooled to room temperature before 12 µl MilliQ water, 2 µl imidazole, 1 µl NADP + ATP solution, and 0.2 µl hexokinase and glucose-6-phosphate dehydrogenase (0.425 and 0.212 U/µl, respectively) were added to each sample for a total reaction volume of 26.2 µl. Samples were homogenized using a vortex and centrifuged at 12,000 rpm for 20 s. Then, the samples were incubated for 5 min at room temperature before the

absorbance of NADPH at λ = 340 nm (A_1) was measured using a NanoDrop (ND-1000, Isogen, The Netherlands). Trehalase (1 µl) was added and samples were again homogenized using a vortex, centrifuged at 12,000 rpm for 20 s, and incubated at room temperature for 5 min before the absorbance of light at λ = 340 nm by NADPH was measured (A_2). Subsequently, the difference in the absorbance of NADPH was calculated (A_2 - A_1). The difference in the absorbance of the blank sample (ΔA_{blank} = A2 - A1) was subtracted from the difference in absorbance of the samples with cysts ($\Delta A_{trehalose} = (A_2 - A_1) - \Delta A_{blank}$). The concentration of trehalose was calculated as follows:

$$[\text{Trehalose}] = \Delta A_{trehalose} \times (MW_{trehalose} / \varepsilon \times d \times 2)$$

where MW is the molecular weight of trehalose (g mol^{-1}), ε is the extinction coefficient of NADPH at 340 nm, d is light path (cm), and 2 represents 2 molecules of D-glucose released from each hydrolysed molecule of trehalose. The amount of NADPH formed is related with the amount of D-glucose and thus with twice the amount of trehalose. Eventually, the amount of trehalose in the whole sample was calculated by multiplying the concentration of trehalose by the reaction volume.

The relationship between the number of viable cysts and the trehalose detected after extraction with the above procedure was determined by quantifying the trehalose content of 1, 5, 10, 15, and 20 cysts of *G. rostochiensis* and of *G. pallida* in reaction volumes of 26.2, 52.4, 78.6, and 104.8 µl. Different reaction volumes were chosen to examine the possible effect of the amount of the reaction volume on the quantity of trehalose detected. For every reaction volume, samples were arranged in a randomized complete block design. Each combination of the cyst number and the reaction volume had 10 replicates. For volumes where results indicated a linear association between the number of eggs and the trehalose content, the best-fit linear regression line was calculated. These experiments were conducted three times (n = 30). In the case of single cysts, the number of eggs was counted to correlate the measured quantity of trehalose to the number of viable eggs (data not shown).

5.2.4 Effect of crushing cysts on the amount of trehalose detected

To determine the effect of crushing cysts on the quantity of trehalose, 10 batches of 1 crushed or non-crushed cyst of each of the PCN were prepared. The trehalose was extracted in a reaction volume of 26.2 µl (as described above). This test was performed three times (n = 30).

5.2.5 Trehalose level in non-viable cyst samples

Because of the quarantine status of PCN, the viability test might also be useful to determine the presence or absence of viable eggs in a sample. To define the threshold below which a trehalose signal cannot be detected in viable eggs, PCN cysts with non-viable eggs (*i.e.*, without trehalose) were used. Non-viable cysts were obtained artificially and from the field. Cultured viable cysts of *G. rostochiensis* and *G. pallida* were pre-soaked in water for 24 h before being killed by incubation at 99°C in MilliQ water for 30 min (van den Elsen *et al*., 2012) and replacing the supernatant (that contains the released trehalose) by MilliQ water. After incubation at room temperature for 2 days, the supernatant was again discarded and the cysts were washed 4 times with MilliQ water. MilliQ water was added to the rinsed cysts, which were then incubated at room temperature for another day. Finally, the supernatant was discarded and the cysts were rinsed 6 times with MilliQ water. The viability of 20 of these cysts of each PCN species was checked for each individual cyst using the trehalose-based method (see above). This was repeated with another batch of 20 cysts of both PCN species (n = 40). Additionally, naturally dead cysts of *G. rostochiensis*, collected from 2 different fields and stored in the lab at room temperature for more than 10 years, were examined. Twenty cysts, 10 from each field, were used separately for the trehalose-based method. This test was performed twice (n = 20). The non-viability of cysts from the field populations and of the heat-killed cysts was confirmed using subsamples of 20 cysts of each category. Both the visual method and the hatching assay (10 weeks) were performed on individual cysts in repeated experiments (n = 40).

5.2.6 Detection threshold of the trehalose-based method

The sensitivity of the trehalose-based method was evaluated by determining the lowest number of viable eggs or cysts that can be detected. Four different experiments were conducted. In experiments 1 and 3, the trehalose content of 1, 2, 3, 4, 5, and 10 eggs (in a reaction volume of 26.2 µl) and of 1, 5, 10, 15, and 20 cysts (in a reaction volume of 104.8 µl) was measured,

respectively (Table 5.1). In experiment 4, to examine the detection of viable cysts of *G. rostochiensis* mixed with non-viable cysts, viable cysts were mixed with heat-killed, non-viable cysts in different proportions (1:19, 5:15, 10:10, 15:10, and 20:10 viable:nonviable cysts) each in a reaction volume of 104.8 µl (Table 5.1). In experiment 2, heat-killed cysts were crushed and non-viable eggs were mixed with viable eggs in proportions of 5 viable eggs mixed with 5, 10, 15, and 20 non-viable eggs, as well as 10 viable eggs also mixed with 5, 10, 15, and 20 non-viable eggs (Table 5.1). Subsequently, the trehalose content was extracted and quantified in a reaction volume of 26.2 µl. Experiments were replicated 10 times and experiments were conducted three times (n = 30). The same four experiments were performed with cysts and eggs of *G. pallida* with the same level of replication and experimental repeats (Table 5.1).

5.2.7 Quantification of trehalose in eggs and construction of a standard curve

The quantity of trehalose released from a cyst depends on the number and viability of eggs in the cyst. Hence, to construct a correlation between the number of viable eggs and the trehalose content (a standard curve), the amount of trehalose was determined in a dilution series of eggs. Cysts from cultures of *G. rostochiensis* and *G. pallida* were crushed in MilliQ water to obtain a stock egg suspension. The number of viable eggs per unit volume was determined based on four 3 ml subsamples as described above. A dilution series of 100, 200, 300, 400, 500, 600, 700, 800, and 900 viable eggs of both *G. pallida* and *G. rostochiensis* was prepared. The trehalose contents of the eggs in these dilution series were determined in reaction volumes of 26.2, 52.4, 78.6, and 104.8 µl. For each reaction volume (treatment) samples were arranged in a randomized complete block design with 10 replicates and the whole experiment was conducted four times for *G. rostochiensis* (n = 40) and twice for *G. pallida* (n = 20). For volumes where results indicated a linear association between the number of eggs and the trehalose content, the best-fit linear regression line was calculated.

5.2.8 Reliability and robustness of the trehalose-based method

The reliability of the trehalose-based method was tested by comparing its efficacy with visual assessment and the hatching assay using individual cysts. For the visual assessment, 20 individual cysts were crushed separately as described above. The total percentage of viable eggs in each cyst was determined. For the trehalose-based method, another 20 individual cysts were

crushed and eggs were released. Then, trehalose was extracted from all eggs obtained from one cyst in a reaction volume of 78.6 µl as described above. The data on trehalose content were converted to the number of eggs based on the equation of the standard curve. The viability of 20 individual cysts was also assessed by determining the hatching percentage after a 10-week exposure of each cyst to the PRD. This experiment was performed twice for both PCN (n = 40).

5.3 Data analysis

For the construction of the standard curve, linear regressions were performed to assess the relationship between the numbers of cysts or eggs and trehalose content, after assumptions of linear regression were tested. The regression lines obtained from the repeated tests were compared with Analysis of Covariance (ANCOVA). When no significant differences were found among the intercept and the slopes of the regression lines, the data of the repeated tests were combined to construct a standard curve. Data of the trehalose content of crushed and non-crushed cysts were subjected to a t-test. The percentage of viable eggs measured by each of the three viability assessment methods was subjected to analysis of variance (ANOVA) to determine the effect of the method on the viability assessment, after assumptions of ANOVA were tested. The differences among the viability assays were analysed with Tukey's Honest Significant Difference (HSD) tests and differences were considered significant at $P < 0.05$. All the analyses were performed using STATISTICA_9 software (Statsoft, OK, USA).

5.4 Results

5.4.1 Determining viability of cultured cysts by hatching assay and visual assessment

The average number of eggs per cyst of *G. rostochiensis* and *G. pallida* was 435 ± 46 and 418 ± 51 eggs, respectively. The final percentage hatch after 14 weeks exposure to PRD was 99% for both *G. pallida* and *G. rostochiensis*. Percentage hatch of J2 from eggs in cysts of *G. rostochiensis* and *G. pallida* over 3 weeks was 49% ± 1.6 and 37% ± 2.5, respectively. After 8 weeks, a period often applied in diagnostic laboratories, the percentage hatch of J2 for *G. rostochiensis* and *G. pallida* had reached 84% ± 1.3 and 84% ± 1.5, respectively.

The visual assessment determined 91% and 88% viability for *G. rostochiensis* and *G. pallida*, respectively.

5.4.2 Quantification of trehalose in cysts and construction of a standard curve

There was no increase in the detected amount of trehalose with increasing number for cysts of both PCN species when the reaction volume was 26.2, 52.4, or 78.6 µl (Figures 5.1 A and C). Similar results were obtained in the repeated tests (data not shown). However, in a reaction volume of 104.8 µl, the concentration of trehalose increased linearly with increasing numbers of cysts of *G. rostochiensis* and *G. pallida* (P = 0.001 and P = 0.01, respectively) (Figures 5.1 A and C). Three regression lines were obtained from the three repeated tests with *G. rostochiensis* cysts in the reaction volume of 104.8 µl: $y = 0.0801x + 2.32$, $R^2 = 0.97$, $P = 0.001$; $y = 0.0481x + 2.12$, $R^2 = 0.85$, $P = 0.04$; $y = 0.0257x + 2.54$, $R^2 = 0.81$, $P = 0.01$. The slope and intercept of these regression lines were different ($P < 0.05$) (Figure 5.1 B), therefore, the data of the three repeated tests were not combined. The same was observed for *G. pallida* with the three regression lines ($y = 0.0381x + 2.59$, $R^2 = 0.89$, $P = 0.01$; $y = 0.0521x + 2.02$, $R^2 = 0.72$, $P = 0.04$; $y = 0.0213x + 2.36$, $R^2 = 0.30$, $P = 0.11$) also being significantly different ($P < 0.05$) (Figure 5.1 D). The results of the trehalose content of different numbers of both PCN cysts revealed that only in high reaction volumes a linear relationship between the number of cysts and the trehalose content was achieved. However, this relationship varied among three repeated tests.

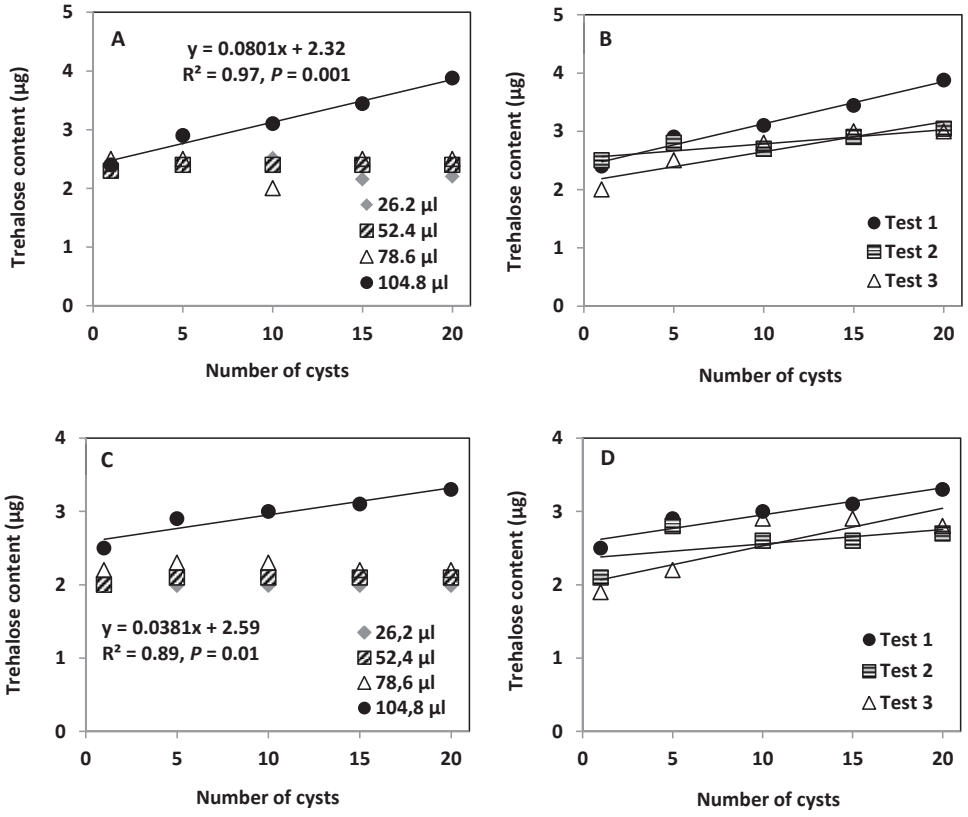


Figure 5.1. Relationship between the numbers of non-crushed cysts and the detected amount of trehalose in different reaction volumes for *Globodera rostochiensis* (A) and *G. pallida* (C) (obtained from one test) and in a reaction volume of 104.8 µl in three repeated tests for *G. rostochiensis* (B) and *G. pallida* (D). The fitted linear regression line corresponds to the relationship between the number of cysts and measured trehalose content in a reaction volume of 104.8 µl. Each data-point is the mean of 10 replicates.

5.4.3 Effect of crushing cysts on the amount of trehalose detected

The quantity of trehalose released from a single non-crushed and crushed cyst was 2.35 ± 0.26 µg and 2.85 ± 0.016 µg for *G. rostochiensis*, respectively, and 2.00 ± 0.61 µg and 3.12 ± 0.12 µg for *G. pallida*, respectively (Figure 5.2). Crushing cysts resulted in higher trehalose concentrations than extracting trehalose from non-crushed cysts for *G. rostochiensis* and *G. pallida* ($F = 39.56$; $df = 1$; $P = 0.001$ and $F = 15.28$; $df = 1$; $P = 0.001$, respectively).

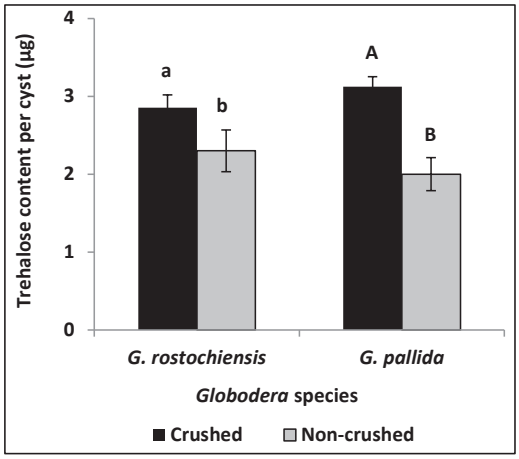


Figure 5.2. Trehalose detected in a crushed and non-crushed cyst of *Globodera rostochiensis* and *G. pallida* in a reaction volume of 26.2 µl. Values are the means of the combined data of three tests (n = 30). Bars marked with a different lower- or uppercase letter are significantly different according to the t-test ($P < 0.05$). Error bars represent the standard deviation of the mean.

5.4.4 Trehalose level in non-viable cyst samples

Both heat-treated cysts and old cysts collected from the field were classified as non-viable by both visual assessment (0%) and hatching assay (0%). The $\Delta A_{trehalose}$ for heat-treated cysts was 0.004 ± 0.002 (n = 40). The $\Delta A_{trehalose}$ for old (=dead) cysts originating from the field was 0.005 ± 0.002 (n = 20). The results for the repeat of the test were similar and the value for $\Delta A_{trehalose}$ varied between 0 and 0.008 in both tests. Based on these results, the threshold value for PCN viability was determined as $\Delta A_{trehalose} = 0.008$; below this value, cysts will be considered non-viable.

5.4.5 Detection threshold of the trehalose-based method

In experiment 1, only 5 and 10 eggs gave a trehalose signal above the threshold level ($\Delta A_{trehalose} = 0.008$) for both PCN species (Table 5.1). In experiment 3, a trehalose signal above the threshold level was detected in all samples containing 1 to 20 cysts. In experiments 2 and 4, viable eggs or viable cysts of both species were always detected despite the presence of non-viable eggs or non-viable cysts with a $\Delta A_{trehalose}$ above the viability threshold. The exception was for *G. rostochiensis* cysts where less trehalose was detected from 5 viable cysts mixed with

15 non-viable cysts (P = 0.01). This same trend was not observed for *G. pallida* cysts (Table 5.1).

Table 5.1. Sensitivity of the trehalose-based method.[a]

Experiment	Viable eggs	Non-viable eggs	Viable cysts	Non-viable cysts	Reaction volume (µl)	[b](A_2-A_1) - ΔA blank (*G. rostochiensis*)	[b](A_2-A_1) - ΔA blank (*G. pallida*)
1	1				26.2	0.0010 ± 0.0012*	0.0015 ± 0.0010*
1	2				26.2	0.0018 ± 0.0011*	0.0015 ± 0.0009*
1	3				26.2	0.0021 ± 0.0022*	0.0021 ± 0.0015*
1	4				26.2	0.0020 ± 0.0012*	0.0022 ± 0.0012*
1	5				26.2	0.0104 ± 0.0007	0.0104 ± 0.0006
1	10				26.2	0.0126 ± 0.0031	0.0129 ± 0.0029
2	5	5			26.2	0.0106 ± 0.0006	0.0106 ± 0.0005
2	5	10			26.2	0.0107 ± 0.0009	0.0106 ± 0.0008
2	5	15			26.2	0.0104 ± 0.0005	0.0107 ± 0.0006
2	5	20			26.2	0.0105 ± 0.0006	0.0105 ± 0.0007
2	10	5			26.2	0.0119 ± 0.0010	0.0118 ± 0.0009
2	10	10			26.2	0.0122 ± 0.0021	0.0125 ± 0.0020
2	10	15			26.2	0.0125 ± 0.0027	0.0126 ± 0.0023
2	10	20			26.2	0.0124 ± 0.0025	0.0123 ± 0.0025
3			1		104.8	0.2811 ± 0.0781	0.2900 ± 0.0812
3			5		104.8	0.4921 ± 0.2123	0.4012 ± 0.2105
3			10		104.8	0.3895 ± 0.1654	0.4184 ± 0.3257
3			15		104.8	0.4813 ± 0.3112	0.4956 ± 0.2987
3			20		104.8	0.4914 ± 0.1839	0.4981 ± 0.2845
4			1	19	104.8	0.2031 ± 0.1102	0.2952 ± 0.0798
4			5	15	104.8	0.2974 ± 0.1014	0.4017 ± 0.1985
4			10	10	104.8	0.3945 ± 0.2679	0.4221 ± 0.3012
4			15	10	104.8	0.4854 ± 0.2134	0.4890 ± 0.1983
4			20	10	104.8	0.5098 ± 0.2387	0.4075 ± 0.2965

[a] Trehalose concentration (mean ± standard deviation) detected in viable eggs or cysts of *G. rostochiensis* and *G. pallida* alone or mixed with non-viable eggs or cysts in different proportions, each containing 30 replicates.
[b] (A_2-A_1) = difference in absorbance of NADPH at λ = 340 nm measured in series 1 to 4; ΔA blank = A_2-A_1 = difference in absorbance of NADPH at λ = 340 nm measured in a blank sample without cysts or eggs.
* indicates below the threshold value for PCN viability (0.008).

5.4.6 Quantification of trehalose in PCN eggs and construction of a standard curve

Extraction of trehalose from eggs of *G. rostochiensis* in a reaction volume of 26.2 µl resulted in increasing trehalose content up to 300 eggs (11.4 eggs per µl reaction volume); the trehalose detected in samples with more eggs was approximately constant, independent of the number of eggs (Figure 5.3 A). When a reaction volume of 52.4 µl was used, this plateau phase was reached at approximately 500 eggs, or 9.5 eggs per µl reaction volume. In reaction volumes of 78.6 and 104.8 µl, a linear relationship between the number of eggs and trehalose content was

observed ($P < 0.05$). However, the regression based on the reaction volume of 78.6 µl gave the best fit ($R^2 = 0.96$, $P = 0.001$) (Figure 5.3 A). Four regression lines were obtained from the four repeated tests with *G. rostochiensis* eggs, y = 0.006 x + 0.65, $R^2 = 0.96$, $P = 0.001$; y = 0.006 x + 0.58, $R^2 = 0.96$, $P = 0.001$; y = 0.007 x + 0.55, $R^2 = 0.95$, $P = 0.002$; y = 0.006 x + 0.59, $R^2 = 0.96$, $P = 0.001$. There was no significant difference between these regression lines (data not shown). Therefore, the data of the repeated tests for *G. rostochiensis* were combined to construct a standard curve described by the equation y = 0.006 x + 0.62 where y = the trehalose content of the eggs and x = the number of viable eggs (Figure 5.3 A).

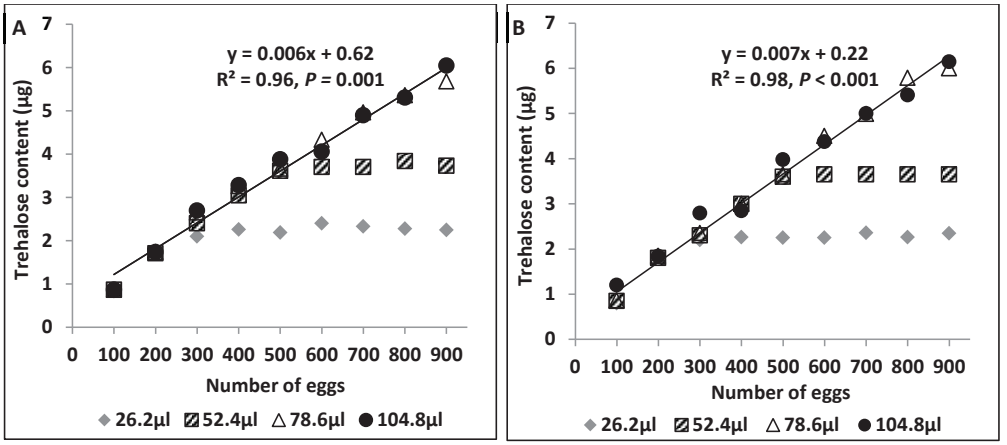


Figure 5.3. Relationship between the number of viable eggs of *Globodera rostochiensis* (A) and *G. pallida* (B) and measured trehalose in different reaction volumes. Values are the means of combined data of four repeated experiments (n = 40) for *G. rostochiensis* and two repeated experiments (n = 20) for *G. pallida*. The fitted linear regression line corresponds to the relationship between the number of eggs and measured trehalose content in a reaction volume of 78.6 µl.

A similar relationship was observed between the trehalose content of *G. pallida* eggs and density in a reaction volume of 78.6 µl and was described by y = 0.007 x + 0.22 ($R^2 = 0.98$, $P < 0.001$) (Figure 5.3 B). This equation was also obtained from the combination of data obtained from two repeated tests. Comparing the slopes and the intercepts of the two regression lines for *G. pallida* (y = 0.007 x + 0.22, $R^2 = 0.98$, $P < 0.001$ and y = 0.007 x + 0.21, $R^2 = 0.98$, $P < 0.001$) also showed no significant difference.

5.4.7 Reliability and robustness of the trehalose-based method

Significant differences were found between the methods for assessing egg viability of cultured cysts of *G. rostochiensis* ($F = 5.68$; $df = 2$; $P < 0.05$). More eggs were classified viable when assessed visually (87.9%) or using the trehalose-based method (86.8%) than with the hatching assay (83.1%) (Figure 5.4). Similar results were obtained with cysts of *G. pallida*: no differences were found between the visual assessment and the trehalose method when assessing the viability of cultured cysts (88 and 86.1%, respectively), whereas, the hatching test identified fewer eggs as viable (84.0%) ($F = 6.24$; $df = 2$; $P = 0.001$) (Figure 5.4).

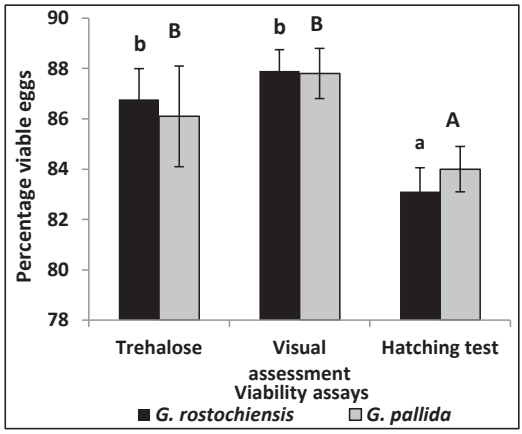


Figure 5.4. Comparison of three methods (trehalose-based method, visual assessment and hatching assay) to measure the viability of eggs of *Globodera rostochiensis* or *G. pallida*. Bars are the means of combined data of two experiments and error bars represent the standard errors of the mean (n = 40). Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

5.5 Discussion

This study showed that the trehalose content of eggs of PCN can be quantified and used to determine the number of viable eggs in a sample. The determination of egg viability of both *G. rostochiensis* and *G. pallida* using the trehalose-based method yielded similar results. Moreover, similar data were obtained from different repeated experiments for both species. Therefore, it can be concluded that the trehalose-based method is a robust tool to determine the viability of PCN species eggs.

More trehalose was detected in crushed cysts than in intact cysts. Part of the trehalose might have been locked up inside the cysts and unavailable for the reaction with trehalase, or perhaps part of the glucose molecules were retained within the cysts and therefore unable to diffuse into the reaction solution. My results showed that the difference in trehalose detected in a crushed and a non-crushed cyst was 0.55 and 1.12 µg for *G. rostochiensis* and *G. pallida*, respectively, corresponding with about 50 and 100 live eggs per cyst. Hence, to attain more precise numbers of viable eggs of PCN present in a sample, it is recommended to crush the cysts to release eggs prior to the trehalose extraction. If not, one will underestimate the population density of viable PCN eggs and consequently, the threat of damage caused by these nematodes.

As the quantification of viable eggs is based on an enzymatic reaction, the reaction volume of the kit (K-TREH 01/09), including the enzyme, needs to be adapted to the substrate concentration (*i.e.*, the number of viable eggs). I observed that about 150 eggs can be retained in 1 µl solution, but an accurate quantification of the amount of trehalose is not possible when the number of viable eggs per µl reaction solution exceeds approximately 10.5 eggs. The insufficient detection of trehalose when there are more than 10.5 eggs per µl reaction can be caused by too low a concentration of trehalase needed to hydrolyse the trehalose present in all the eggs to D-glucose, or the inhibition of the trehalase activity due to biochemical changes in the reaction solution. It has been shown that trehalase is inactivated in a suboptimal pH and temperature (de Andrade *et al*., 2000). Nevertheless, regarding the experimental set up, the effect of temperature cannot be accounted for it.

The threshold value for egg viability established in my experiment was $\Delta A_{\text{trehalose}} = 0.008$, whereas van den Elsen *et al*. (2012) presented a threshold value of 0.0094 for a non-viable PCN population. Additionally, based on the trehalose signal obtained from dead cysts, I defined the detection limit as 5 viable eggs. Van den Elsen *et al*. (2012) showed that in the case of field populations, the detection limit is 10 viable PCN eggs in a background of 25 nonviable PCN cysts. The number of eggs in young PCN cysts in the field varies on average between 100 and 450 eggs, so 5 eggs is an acceptable threshold and the trehalose method can be considered as very sensitive. However, when dealing with statutory samples where no live cysts should be present, or when evaluating disinfestation or other control measures where 100% non-viability is required, this threshold should be kept in mind. Nevertheless, other viability assays have similar

limitations. Therefore, the restrictions of each viability assay should be considered when selecting a method for a specific purpose.

This study showed that the presence of non-viable eggs in the sample does not reduce the detection of viable eggs, which was the case for both PCN species. This finding makes the trehalose-based method a sensitive technique when assessing viability of encysted eggs from fields. However, it should be noted that this was not always the case when using cysts instead of eggs. In two of the three repeated experiments, 5 viable cysts of *G. rostochiensis* in a background of 15 non-viable ones yielded less trehalose than 5 viable cysts alone, thereby influencing the sensitivity of the trehalose method. Yet, with the other proportions of dead and viable cysts of *G. rostochiensis* and with all cyst combinations of *G. pallida* the presence of non-viable cysts did not influence the outcome of the trehalose method. More investigation is needed to address this difference. Nevertheless, this observation with a combination of viable and non-viable cysts again recommends the detection of trehalose in eggs freed from cysts.

In accordance with van den Elsen *et al*. (2012) and Beniers *et al*. (2014), a relationship was found between the egg viability and the trehalose content. A standard curve was constructed for both PCN species to have a precise correlation between the number of viable eggs and trehalose content. This standard curve allows for the enumeration of viable eggs in a sample in a fast and objective way. The only drawback of the method is that the concentration of viable eggs should not exceed 10.5 eggs per µl. This requires a preliminary estimation of the number of eggs by quick microscopic observations of crushed cysts and their content, so that -in case the 10.5 eggs per µl is exceeded- an appropriate dilution of the egg suspension can be made prior to measuring trehalose content. Beniers *et al*. (2014) also reported a saturation phase during the quantification of trehalose. These authors constructed one standard curve for both PCN species; however, in this study, there was a significant difference between the regression lines describing the relationship between the number of viable eggs and the trehalose content for *G. rostochiensis* and that for *G. pallida*. Moreover, different from Beniers *et al*. (2014), trehalose was extracted from a dilution series of eggs in higher reaction volumes which resulted in less variability in the trehalose measurement. Beniers *et al*. (2014) also examined the validity of the method using cysts treated with allylisothiocyanate. They noted that the trehalose-based method overestimated the PCN viability when eggs were exposed to this chemical for one week and killed. However,

this method was used to determine the viability of encysted eggs treated with amendments and inundation (see chapter 6 and 7) and it proved to be a reliable viability assessing method.

Although I did not observe fungal growth in the eggs nor checked for bacterial contamination, it should be noted that trehalose is the most widely distributed disaccharide in fungi (Thevelein, 1984) and can also be found in different classes of prokaryotes (Arguelles, 2000). Therefore, a positive signal might be possible from dead eggs because of fungal or bacterial contamination. In this case, trehalose from fungi or other sources can be released by sonication of samples for 120 s (Hallsworth & Magan, 1994), a technique that does not influence the permeability of the eggshell of PCN (Y. Valdes, pers. comm.). The extracted trehalose is then degraded by trehalase and measured. Subsequently, the trehalose content of PCN eggs can be released by their incubation at 99°C for 30 min.

Currently, a range of tests are used for assessing viability of eggs and J2 of plant-parasitic nematodes in different laboratories. Visual evaluation of the viability of eggs and J2 of both *Globodera* species is a simple means and recognized as a valid tool (OEPP/EPPO, 2013) for diagnostic laboratories. Visual assessment, nevertheless, is labour intensive and time consuming when many samples need to be evaluated, however, its largest disadvantage is that it requires well trained and experienced personnel as it involves subjectivity. In my trial, the trehalose-based method gave similar results to those obtained with visual assessment. Meyer *et al*. (1988) used different stains to estimate egg viability of *Heterodera glycines*. They concluded that some eggs could not be readily classified when using the stains and the results were not always consistent. Meldola's blue is the most popular and reliable staining agent to determine egg viability (Kroese *et al*., 2011) and it has also been used to assess the egg viability of *Globodera* when evaluating management measures (Twomey *et al*., 2000) and conducting surveys of regulatory significance (Rott *et al*., 2010). Currently, Meldola's blue is not available for purchase.

Determination of viability by a hatching assay also requires a lot of time (weeks) and is subject to many factors such as diapause and the quality of the PRD (Kroese *et al*., 2011). The results of this study showed that assessing the viability of both PCN species with a hatching assay lasting 10 weeks led to fewer live eggs than the other two methods. This slightly lower

viability (about 2%) obtained with the hatching assay could be attributed to some unhatched J2, which were viable even though they did not hatch. Perry & Moens (2011) stated that a small percentage of J2 of *G. rostochiensis* do not hatch within a certain period due to diapause and persist in the field for many years before they hatch. An assessment of PCN viability for statutory purposes (field sampling for seed potato production, soil adhering to imported potato tubers) is usually needed in a short period of time. Hence, diagnostic labs performing hatching tests in less than 10 weeks can miss out some live eggs still present inside cysts. Also, when viability is measured for large numbers of samples, the trehalose-based method is much faster than a long-lasting hatching assay.

Compared with the existing viability methods, the trehalose-based method is a feasible, sensitive, and inexpensive technique. A single trehalose detection kit can be used for 1,000 to 10,000 viability tests at a cost of $0.03 to $0.27 per sample depending on the reaction volume. The trehalose-based method is also a standardized and objective technique requiring little training. This approach is likely to be a fast tool for assessing PCN egg viability when dealing with a large number of samples. The qualitative trehalose-based method developed by van den Elsen *et al.* (2012) has been added to the laboratory techniques recommended to determine the viability of PCN eggs (OEPP/EPPO, 2013) but the reproducibility of this optimised quantitative technique needs to be evaluated by different laboratories.

Chapter 6

The effects of soil amendments on the survival and reproduction of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*)

6.1 Introduction

Potato cyst nematodes (PCN) are managed by integrating different control options, *e.g.*, crop rotation, nematicides and resistance (Hockland *et al*., 2012). However, both the increasing environmental concern for nematicides resulting in the withdrawal of several chemicals from the market, and the limited number of potato cultivars resistant to *G. pallida*, demand alternative management strategies. In view of this, the potential value of the application of soil amendments for managing PCN is worth to be examined.

Annually, a large amount of agricultural and agro-industrial by-products, all considered waste, is generated. Many of these products can be recycled for agricultural use, *e.g*., as fertiliser or C-rich soil amendment. Organic amendments can also reduce disease incidence caused by plant pathogens including nematodes (Abawi & Widmer, 2000; Akhtar & Malik, 2000; Lazarovits *et al*., 2001; Renčo *et al*., 2007; Oka, 2010). Nematode suppression by soil amendments is caused by different mechanisms including *i*) improvement of the physical and chemical properties of the soil, which may have an adverse influence on hatching, mobility and survival, *ii*) the release of nematicidal compounds by the organic material, *e.g*., organic acids, phenolic compounds and ammonium, *iii*) improvement of plant growth and *iv*) changes in biological properties of the soil.

It is shown that organic amendments affect the microbial community of the soil, *e.g*., bacteria, fungi and free-living nematodes (Oka, 2010). Many of these organisms, or their metabolites, are beneficial for plant growth or antagonistic towards plant pathogens including nematodes (Stirling, 1991; Viaene & Abawi, 2000; Arancon *et al*., 2003). Introduction of some specific microorganisms such as bacteria, fungi and mycorrhiza induces plant systemic resistance to nematodes (Reitz *et al*., 2000; Kluepfel *et al*., 2002; Dababat & Sikora, 2007; Sikora *et al*, 2008; Elsen *et al*., 2008).

Animal manures, crab shells, and several kinds of compost and plant extracts, have been studied for their potential to manage plant-parasitic nematodes. Tian *et al*. (2000) found that soil amended with chitin may improve the development of nematode antagonistic soil fungi and bacteria, leading to suppression of the soybean cyst nematode, *Heterodera glycines*. It was also shown that swine manure contains a large amount of proteins that can be converted to volatile

fatty acids (VFA) and ammonium (NH_4^+), both having nematicidal activities (Chitwood, 2002). It was also reported that swine manure inhibited hatch and movement of J2 of *H. glycines* towards the host root (Reynolds *et al*., 1999). Similarly, Xiao *et al*. (2008) reported that VFA-enriched swine manure inhibited *H. glycines* hatch and root penetration; exposure of hatched J2 to the swine manure for eight hours caused more than 90% mortality. Anaerobically digested cattle slurry reduced the densities of *Meloidogyne incognita* in tomato (Jothi *et al*., 2003). Renčo *et al*. (2007) examined the effects of five composts of different origin (plants, animals and fungi) on *G. rostochiensis* on potato in pot experiments. All tested composts reduced the number of eggs and J2. Many plant extracts have also shown nematicidal effects (Chitwood, 2002). Extracts from several *Allium* spp. have shown nematicidal activities (Auger & Thibout, 2005; Arnault *et al*., 2004). Different sulphur-containing compounds, *e.g*., dipropyl disulphide (DPDS), diallyl disulphide (DADS) and dimethyl disulphide (DMDS), generated by degradation of *Allium* plants (leek, onion and garlic) are toxic to nematodes. The toxicity of these disulphides is shown to be equal to that of methyl bromide for nematodes and insects (Arnault *et al*., 2004).

Few studies have investigated the effects of the addition of biochar on soil inhabiting nematodes. Biochar is produced by thermal treatment of biomass at oxygen deficiency, *e.g*., by pyrolysis or gasification. It can be used as a soil amendment (Lehmann & Joseph, 2009) and sequester carbon (C) to mitigate climate change (Lehmann *et al.,* 2006). Biochar has a high specific surface area (400-800 $m^2\ g^{-1}$) providing a habitat for soil microorganisms (Fischer & Glaser, 2012). It has also been shown to change the composition and abundance of the soil biological community (Pietikäinen *et al.,* 2000). However, Nelissen *et al*. (2015) reported no significant influence of the addition of biochar to the soil on the total amount of microbial biomass.

Most research on soil amendments has focused on their influence on PCN during the potato crop; little effort has been placed on their effect on the survival and viability of PCN between successive host crops. However, the latter type of research may yield extra information on the factors affecting decline rates of PCN. Schomaker & Been (2013) reported a mortality rate of 69% for *G. rostochiensis* in the absence of the host in the first year after a potato crop; in subsequent years they observed 20-30% mortality rate. In view of this, I evaluated in pot experiments the suppressive effect of different soil amendments on *G. rostochiensis* and *G.*

pallida in the presence of potato as well as in its absence. I focused on amendments that were locally available and economically acceptable by farmers, but also on crab shell compost and new products like biochar. The specific objectives of this study were *i*) to determine the effect of a series of soil amendments on the survival of PCN in the absence of a host, *ii*) to examine the impact of the amendments on the reproduction of PCN on potato, *iii*) to understand the modes of action of these soil amendments in PCN suppression by looking at their effect on hatching, movement, host finding ability and root penetration of juveniles, and *iv*) to unravel mechanisms of nematode suppression by soil chemical and biological analyses.

6.2 Materials and methods

6.2.1 Nematodes

The populations of *G. rostochiensis* (Kruishoutem, Belgium) and *G. pallida* (Chavornay, Switzerland) used in this experiment were obtained from stock cultures (see section 3.1). Cysts were maintained on potato cv. Désirée under greenhouse conditions (20-25°C, 16 h light). Cysts were stored for 4 months at 4°C to overcome the diapause. The egg content of the harvested cysts of *G. rostochiensis* and *G. pallida* was 435 ± 46 and 418 ± 51 eggs, respectively.

6.2.2 Amendments

The following amendments were used in the experiment: pig slurry, cattle slurry, crab shell compost, nitrogen fertiliser in the form of ammonium nitrate (NH_4NO_3) (0.068 g kg^{-1} soil), wood chip compost (made from 30% straw, 18% wood chip, 23% poplar bark, 14% maize straw and 14% leek residue), Romchar (0.3% and 1% V/V) (a type of biochar produced during slow pyrolysis of hard and soft wood at 480°C), Romchar-blended wood chip compost, Romchar-blended pig slurry and Romchar-blended crab shell compost. The blended amendments consisted of 0.3% Romchar (Table 6.1). The two slurry-amended soils were collected from the long-term experimental site BOPACT at the Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium, immediately after the slurries were applied to the soil (57% sand, 37.7% loam, 5.3% clay and 0.67% organic carbon). The amount of pig slurry, cattle slurry, nitrogen fertiliser and composts applied contained the maximum nitrogen doses (170 kg N ha^{-1}) permitted by the European legislation (91/676/EEC) (Anon., 1991; Anon., 2011).

Table 6.1. Amount of amendments applied in the pot experiments with their composition and amounts of nutrients and organic matter.

		Amendment					
Parameters	**Unit**	**Pig slurry**	**Cattle slurry**	**Crab shell compost**	**Wood chip compost**	**Romchar 1%**	**Romchar 0.3%**
Fresh application rate	$g.kg^{-1}$ dry soil	12.7	24.7	4.1	31.9	10.0	3.0
Fresh application rate	$ton.ha^{-1}$	24.7	48.2	8.0	62.1	19.5	5.9
Dry matter (DM)	%/fresh	8.7	8.2	88.7	39.1	76.2	76.2
C:N	-	4.6	10.6	10.7	33.2	170.3	170.3
pH-H_20	-	7.9	8.3	7.9	8.6	8.6	8.6
Nutrients							
Organic matter	%/DM	65.3	82.1	46.3	41.8	90.9	90.9
C	%/DM	NA	NA	NA	NA	75.1	75.1
Total N	%/DM	7.9	4.3	2.4	0.7	0.4	0.4
NO_3-N	$g.kg^{-1}$ DM	< 0.01	< 0.01	0.01	0.01	< 0.01	< 0.01
NH_4-N	$g.kg^{-1}$ DM	55.2	23.2	0.21	0.01	< 0.01	< 0.01
P	$g.kg^{-1}$ DM	20.7	7.9	10.7	1.5	0.54	0.54
K	$g.kg^{-1}$ DM	59.8	44.3	5.1	6.5	4.6	4.6
Ca	$g.kg^{-1}$ DM	35.7	12.6	189.5	12.3	15.4	15.4
Mg	$g.kg^{-1}$ DM	23.2	6.4	10.8	1.7	1.2	1.2
Nutrient application rate							
C	$g.kg^{-1}$ dry soil	0.40	0.92	0.93	2.89	3.178	0.9
Total N	$g.kg^{-1}$ dry soil	0.09	0.09	0.09	0.09	0.030	0.01
NO_3-N + NH_4-N	$g.kg^{-1}$ dry soil	0.06	0.05	< 0.01	< 0.01	< 0.01	< 0.01
P	$g.kg^{-1}$ dry soil	0.02	0.02	0.04	0.02	0.000	0.00
K	$g.kg^{-1}$ dry soil	0.07	0.09	0.02	0.08	0.02	0.01

6.2.3 Effect of amendments on the viability of *G. rostochiensis* and *G. pallida*

The amendments were thoroughly mixed with soil collected from the experimental site at ILVO and added to 2.5-L pots. Only the crab shell compost was mixed with the soil and incubated at room temperature for 8 months prior to use to stimulate the growth of the chitinolytic bacteria in the soil during the incubation period. Non-amended soil served as a control. Batches of 14 cysts of *G. rostochiensis* or *G. pallida* were placed in retrievable nylon mesh bags and placed at a depth of 6 cm in 4 pots (replicates) of every amendment. Pots were left outside for 16 weeks in a completely randomized design and were exposed to prevailing temperature conditions to simulate field conditions. Each treatment had 4 replicates. Every pot received 3 bags containing 14 cysts; they served to estimate the viability of the cyst content at 8, 12 and 16 weeks after soil infestation (WAI), respectively. At each time-point, one bag was

retrieved and 7 cysts were used for visual assessment of the viability (OEPP/EPPO, 2013) and the other 7 cysts for trehalose-based viability determination (see chapter 5). An extra bag containing 20 cysts was placed in each pot to estimate the viability of encysted eggs by a hatching assay 16 WAI. The viability of the content of these 20 cysts was assessed by determining the hatching percentage over a period of 10 weeks exposure to potato root diffusate (PRD) (Turner *et al*., 2009). Three weeks after the start of the experiment, a non-destructive 200-cm^3 soil sample was taken from each pot to search for the spontaneously hatched J2 in the soil. The juveniles were extracted from the soil using an automated zonal centrifuge (Hendrickx, 1995) (see section 3.2.2). The whole experiment was performed twice in consecutive years during spring/summer, in pots left outside in the rain shelter.

6.2.4 Viability tests

Three methods were used to estimate the viability of the cyst content. For the visual determination (OEPP/EPPO, 2013), seven cysts of *G. rostochiensis* or *G. pallida* were soaked in water for 24 h at 21°C and then crushed using a cyst crusher. The volume of the obtained egg suspension was increased to 10 ml of which three subsamples of 3 ml were drawn. The viability of eggs and J2 was determined using a dissecting microscope (100 ×). Damaged and empty eggs with non-smooth eggshells, and shrivelled disintegrated J2 with no clear lip region or stylet, were considered dead (OEPP/EPPO, 2013).

For the trehalose-based method, a homogenized egg suspension was obtained after crushing 7 cysts (see visual determination). Then, 4 subsamples were prepared for the trehalose extraction in a reaction volume of 78.6 µl. The trehalose present in the eggs was measured using a detection kit (K-TREH 01/09, Megazyme International Ireland Ltd., Wicklow, Ireland) and following the protocol defined by van den Elsen *et al.* (2012) (see chapter 5). The obtained data on trehalose content of the eggs of each species were converted to the number of viable eggs based on the standard curves constructed in chapter 5.

For cysts retrieved after 16 weeks, the viability was also assessed by determining the hatching percentage after a 10-week exposure at 21°C to the PRD, which was collected from cv. Désirée following the protocol described by Turner *et al*. (2009) (see section 3.3). The hatched J2 were counted weekly over a period of 10 weeks and PRD was renewed at every counting

event. After 10 weeks, all cysts were crushed and the released viable unhatched juveniles were counted. This number was used to calculate the percentage of the total cyst content that had hatched during the assay.

6.2.5 Effect of pre-exposure of *G. rostochiensis* and *G. pallida* cysts to amendments on hatching of J2

The hatching data from the hatching assay conducted to determine the viability of the cyst content (mentioned above) were also used to study the effect of the amendments on the hatching process from cysts that had been exposed for 16 weeks in the absence of a host.

6.2.6 Effect of pre-exposure of *G. rostochiensis* and *G. pallida* cysts to amendments on infectivity of J2

The infectivity of J2 hatched from cysts exposed to amended soil for 16 weeks was determined. Sprouting potato tubers (cv. Bintje) were planted in 0.5-L closed containers filled with 200 g sterilized sand and 30 ml water and kept in the dark at 21°C (see section 3.5). When potato roots had formed, 1000 freshly hatched J2 were added to each pot. Ten days later, roots were gently removed, washed and nematodes that had penetrated the roots were stained with acid fuchsin and counted (Byrd *et al*., 1983) (see section 3.6).

6.2.7 Effects of amendments on the reproduction of *G. rostochiensis* and *G. pallida*

The amended soils and non-amended soil (control) mentioned above were also added to 4-L pots. One sprouted potato tuber (cv. Bintje) was planted in each of the pots and batches of 33 cysts of *G. rostochiensis* or 34 cysts of *G. pallida* were placed in retrievable nylon voile bags at a depth of 6 cm beneath the tuber. The initial population density was 3.6 eggs $(cm^3\ soil)^{-1}$, for *G. rostochiensis* as well as for *G. pallida*. Each treatment had four replicates (pots) arranged in a completely randomised design.

Sixteen weeks after planting, plants were gently harvested. A 500-cm^3 soil sample was taken from homogenised soil of each pot to determine the nematode reproduction rate. Cysts were extracted from soil using a Seinhorst elutriator (Seinhorst, 1964) (see section 3.2.1). The extracted cysts were counted and then crushed to count the encysted eggs and J2. This

experiment was performed twice, in two consecutive years, in the same conditions as the experiment on effects of amendments on cyst viability in the absence of a host plant (as described above).

6.2.8 Mechanisms of suppression of *G. rostochiensis* and *G. pallida*

6.2.8.1 Effects of amendments on hatching of *G. rostochiensis* and *G. pallida*

To study the effect of amendments on hatching of PCN, PRD was collected four weeks after planting potato cv. Désirée in non-amended soil and soils mixed with amendments mentioned above, following the protocol described by Turner *et al*. (2009) (see section 3.3). All soils were the same as the soil used in the previous experiments, originating from the experimental site. PRD was stored at -20°C until use. Batches of 20 cysts of *G. rostochiensis* or *G. pallida* were soaked in water for 48 h before they were exposed to PRD collected from non-amended soil (control) and amended soils (see section 3.4). Four replicates were used for each treatment. The samples were maintained in dark at 21°C in a completely randomized design. The number of hatched J2 was counted weekly over a period of 10 weeks; the solution was renewed at every counting event. After 10 weeks, all cysts were crushed and the number of unhatched juveniles inside the cysts was determined to calculate the percentage of the total cyst content that had hatched during the assay. The hatching assay was performed twice.

6.2.8.2 Effects of amendments on J2 migration of *G. rostochiensis* and *G. pallida*

To examine the effects of amendments on migration of J2 of *G. rostochiensis* and *G. pallida* in soil, a modification of the sand column assay developed by Clarke and Hennessy (1984) was used. PVC tubes (5 cm long and 1 cm diam.) sealed at one end with a 45µm-aperture nylon mesh were filled with sterilised river sand and saturated with PRD collected from each of the amended soils. The PRD from non-amended soil and water served as controls. The PVC tubes were placed in 20-ml glass bottles containing 2 ml of the corresponding solution used for saturation of the sand. One hundred freshly hatched J2 of one of the PCN species were added on top of the sand column. The bottles with tubes were incubated in the dark at 21°C for 48 h. The number of J2 that had migrated through the sand column into the glass bottles was then counted

and the percentage of migrated J2 was calculated. Each treatment had 4 replicates and the bioassay was performed twice.

6.2.8.3 Effects of amendments on infectivity of J2 of *G. rostochiensis* and *G. pallida* on potato

The effect of the amendments on infectivity of encysted juveniles of both PCN species was determined in 1.5-L pots. Sprouted tubers of the potato cv. Bintje were planted in pots filled with the amended soils or non-amended soil (control). At the same time, batches of 10 cysts of *G. rostochiensis* or 11 cysts of *G. pallida* were placed in retrievable nylon voile bags in each pot beneath the tuber. Pots were arranged in a completely randomized design with 3 replicates for each treatment. Plants were harvested after 10 days, roots were washed, blotted dry, weighed and cut into 1-2 cm pieces. A subsample of 5 g root was taken and juveniles inside the roots were stained with acid fuchsin (Byrd *et al*., 1983).

The same experiment was conducted with freshly hatched J2 instead of cysts in order to discriminate between the possible suppressive effects of amendments on hatching of J2 and their infectivity. Batches of 3000 freshly hatched J2 of *G. rostochiensis* or *G. pallida* were added into 5 holes made 6 cm deep in the soil around the tuber. The experiment was repeated once.

6.2.8.4 Chemical analysis of soil amendments

After incorporation of the amendments into the soil and at the end of the experiment of the effect of amendments on the reproduction of PCN, a 500-g soil sample was collected from each pot (4 replicates) for chemical analysis (pHKCl, dry matter, nitrogen, ammonium nitrogen, phosphorus, C:N ratio, soil organic carbon (SOC), calcium, potassium and magnesium). Samples of 80-g soil were also taken from each pot one week after incorporation of amendments to determine the content of ammonium, nitrate and the mineral nitrogen.

Prior to chemical analysis, samples were divided into three sub-samples. One sub-sample was immediately used for determination of soil pH and mineral nitrogen (N). The pH was measured potentiometrically in a 1:5 soil:KCl (1M) extract according to ISO 10390. Soil mineral N (NO^{3-}-N + NH^{4+}-N) was determined in a 1M KCl extract according to ISO TS14256-1:2003 with a Skalar San++ continuous flow analyser. The second and third sub-samples were oven

dried at 45°C and 70°C, respectively. The samples were ground in a mortar and passed through a 2-mm and 250-µm sieve, respectively. The second subsample was used to assess the ammonium lactate extractable P, Ca, Mg and K (P-AL, Ca-AL, Mg-AL and K-AL). The extraction of the soil was performed with ammonium lactate (extraction ratio 1:20) in dark polyethylene bottles. Bottles were shaken for 4 hours. The suspension was then filtered in dark polyethylene bottles that were stored at 4°C until analysis. P-AL, Ca-AL, Mg-AL and K-AL were analysed at 770 nm and 214 nm, respectively, using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian Vista-Pro) with an axial torch. Soil organic carbon (SOC) and total N were measured on the third subsample by dry combustion at 1050°C using a Skalar Primacs SLC TOC (Total Organic Carbon) analyser (ISO 10694) and at 950°C using a Thermo Flash 4000 N-analyser (ISO 13878), respectively.

6.2.8.5 Phospholipid fatty acid (PLFA) composition of the soil microbiota

At the end of the experiment studying the effect of amendments on the reproduction of PCN, a 100-g soil sample was taken from each pot and stored at -20°C until freeze-dried to determine the biomass and composition of the microbial community by analysing phospholipid fatty acid (PLFA) profiles of soil (Denef *et al.,* 2007). Total lipids were isolated from 6 g freeze-dried soil using phosphate buffer, chloroform and methanol at a 0.9:1:2 ratio. Neutral, glycol-and phospholipids were separated by solid phase extraction eluting, respectively, chloroform, acetone and methanol. Phospholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs).

FAMEs were analysed with a capillary gas chromatograph-flame ionisation detector (Perkin Elmer Clarus 600, Perkin Elmer, Waltham, USA) with a col-elite-2560 column (100 m length x 0.25 mm ID, 0.25 µm film thickness, Perkin Elmer). The temperature program started at 75°C, followed by a heating rate of 10°C min^{-1} to 180°C and followed by a final heating rate of 2°C min^{-1} to 240°C. PLFAs were identified and qualified from the retention time and response factor of each FAME in the external FAME and BAME mix (Sigma Aldrich, St Louis, MO, USA). The C values were corrected using a working standard C19:0.

The abundance of individual PLFAs was calculated in absolute C amounts (PLFA-C, C_x (nmol g^{-1})) based on the concentrations in the liquid extracts using the following formula:

$$C_x[\text{nmol g}^{-1}] = \frac{\text{A}_\text{x} \,.\, \text{c}_\text{i}[\mu\text{g}] \,.\, 1000}{\text{A}_\text{i} \,.\, \text{W [g]} \,.\, \text{M [}\mu\text{g mol}^{-1}\text{]}}$$

where C_x is the concentration of the fatty acid studied, A_x is the peak area of the fatty acid studied, A_i is the peak area of the internal standard, c_i is the absolute amount of internal standard in the vial (µg), W is the amount of soil (g), M is the molecular weight of the fatty acid (µg mol^{-1}).

The fatty acids 10Me-C16:0, 10Me-C18:0 and 10Me-C17:0 were used as indicators of actinomycetes. C18:1c11, C19:0cy, C17:0cy, C16:1C9 and C16:1t9 were chosen to represent gram negative bacteria and a-C15:0, i-C16:0, i-C17:0 and i-C15:0 for gram positive bacteria. Fatty acids C14:0, , C15:0, C16:0, , C17:0 and C18:0 were selected as indicators of non-specific bacteria, C18:2n9 and C18:1c9 for fungi and C16:1c11 for arbuscular mycorrhizal fungi (Nelissen *et al*., 2015).

6.3 Data analysis

The percentage of viable eggs measured by the visual assessment and the trehalose-based method was subjected to factorial ANOVA to determine the effects of the method of viability assessment, amendments, the exposure time and species. Data were arcsine-transformed before being subjected to ANOVA. The non-transformed data were used to produce the graphs. The data of the percentage of viable eggs determined by the aforementioned methods and the hatching assay, 16 weeks after the incorporation of amendments into the soil, were also subjected to ANOVA to determine the differences among the three viability assessing methods. The number of cysts, and encysted eggs and J2 in the experiment of the effect of amendments on reproduction, the data of the migration assay of J2, the number of J2 that penetrated the roots in the infectivity assay, and the results of the soil analyses (chemical and PLFA analyses) were log-transformed when necessary to improve the normality and homogeneity of variation and analysed using ANOVA.

For the hatching assay, the logistic model, y = c/(1+(exp(−b×(time−m)))) was used to fit the hatching data from each treatment, where the cumulative percentage hatch obtained in time (y) is described by the parameters m (time at which 50% hatch is reached), b (hatching rate) and

c (final percentage hatch) (Oude Voshaar, 1994). These parameters were calculated for each replicate of all treatments.

In all analyses, the differences among treatments were compared with Tukey's Honest Significant Difference (HSD) tests and were considered different when $P < 0.05$. All the analyses were performed using STATISTICA_9 software (Statsoft, OK, USA).

6.4 Results

6.4.1 Effect of amendments on the viability of *G. rostochiensis* and *G. pallida*

No significant difference was observed between the visual assessment and the trehalose-based method in assessing the viability of *G. rostochiensis* and *G. pallida* 8, 12, and 16 weeks after the exposure of cysts to amendments ($P = 0.12$, $P = 0.20$ and $P = 0.13$ after 8, 12, and 16 weeks, respectively for *G. rostochiensis* and $P = 0.10$, $P = 0.11$ and $P = 0.16$ after 8, 12, and 16 weeks, respectively for *G. pallida*). Therefore, the effect of the amendments on viability was evaluated with the average data obtained with both methods. The estimate of viability of eggs was significantly influenced by the viability assessing methods (visual assessment, the trehalose-based method and a hatching assay) 16 weeks after the exposure of cysts to amendments. The hatching assay defined fewer viable eggs compared with the other two methods ($F = 15.27$; $df = 2$; $P = 0.003$).

Eight weeks after the start of the experiment, the estimated viability for *G. rostochiensis* eggs was 97.5% in non-amended soil. However, the viability of encysted eggs of *G. rostochiensis* was significantly reduced in soil amended with pig slurry (13%) and crab shell compost (13%) followed by pig slurry mixed with biochar (12%), wood chip compost (11.8%) and nitrogen fertiliser (10%) ($F = 4.61$; $df = 10$; $P < 0.001$) (Figure 6.1 A).

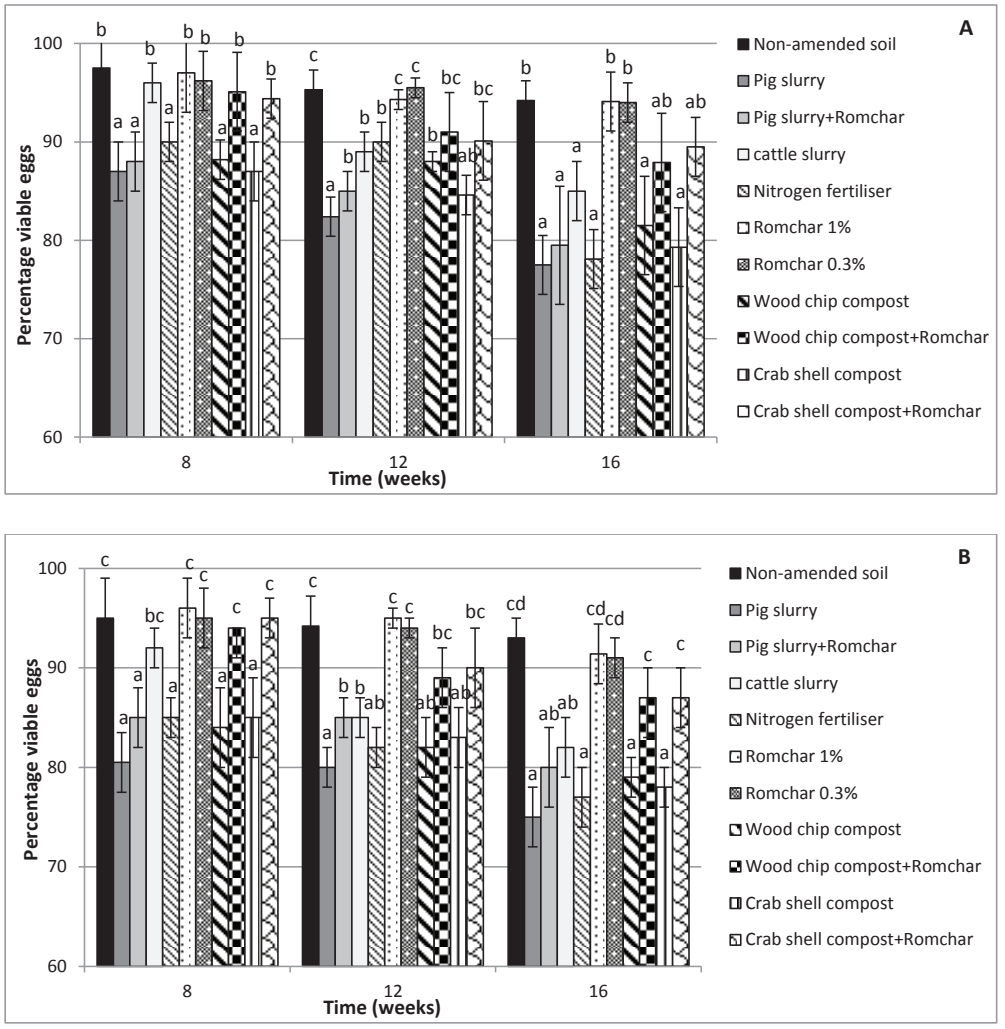


Figure 6.1. Percentage viable eggs of *Globodera rostochiensis* (A) and *G. pallida* (B) in pot experiments at different time intervals after treatment with soil amendments at 170 kg N ha^{-1}. Different letters represent significant differences in the number of viable eggs according to Tukey's Honest Significant Difference test ($P < 0.05$).

Assessment of the viability of encysted eggs 12 weeks after the start of the experiment revealed significant reductions in egg viability in soil mixed with amendments mentioned above and also in soil mixed with cattle slurry ($F = 3.59$; $df = 10$; $P = 0.003$). Sixteen weeks after exposure of the cysts to the amendments, the viability assays showed that the percentage viable eggs of *G. rostochiensis* was significantly reduced in soil amended with pig slurry (22.5%) and

nitrogen fertiliser (21.9%) followed by crab shell compost (20.7%), pig slurry mixed with biochar (20.5%), wood chip compost (18.5%) and cattle slurry (15%) (Figure 6.1 A) ($F = 9.18$; $df = 10$; $P < 0.001$). There was no significant difference in the number of viable eggs 16 weeks after incorporation of other amendments (Figure 1 A). The effect of amendments on viability of *G. rostochiensis* eggs did not depend on the exposure time, as the interaction 'amendments' × 'exposure time' was not significant ($F = 0.62$; $df = 20$; $P = 0.89$). However, the exposure time had a significant effect on the number of viable eggs ($F = 25.79$; $df = 2$; $P < 0.001$): viability of *G. rostochiensis* eggs was reduced with increasing exposure time.

The viability of eggs and J2 of *G. pallida* was also significantly reduced in soil amended with pig slurry, wood chip compost, nitrogen fertiliser, crab shell compost, and pig slurry mixed with biochar 8 and 12 weeks after incorporation of the amendments into soil ($F = 10.14$; $df = 10$; $P < 0.001$ and $F = 9.12$; $df = 10$; $P < 0.001$, respectively) (Figure 6.1 B). The other amendments had no significant effect on viability of *G. pallida* (Figure 6.1 B). The effects of amendments on the survival of encysted eggs of *G. pallida* 16 weeks after exposure was similar to those of *G. rostochiensis* ($F = 7.56$; $df = 10$; $P < 0.001$). Pig slurry, nitrogen fertiliser, crab shell compost, wood chip compost, pig slurry mixed with biochar and cattle slurry reduced the egg viability of *G. pallida* by 25%, 23%, 22%, 21%, 20% and 18%, respectively. The number of viable eggs decreased with exposure time ($F = 25.60$; $df = 2$; $P < 0.001$). However, the interaction 'amendments' × 'exposure time' was not significant ($F = 0.50$; $df = 20$; $P = 0.96$).

There were differences between the viability of *G. rostochiensis* and *G. pallida*, ($F = 18.94$; $df = 1$; $P < 0.001$). However, the influence of the amendments on the egg viability was similar for both species (interaction 'amendments'× 'species': $F = 0.51$; $df = 10$; $P = 0.88$). The decrease of egg viability with time was also independent of the species (interaction 'species' × 'time' $F = 0.46$; $df = 2$; $P = 0.63$). As stated above, for both *Globodera* species separately, the effect of the amendments was independent of exposure time (interaction 'amendments' × 'time': $F = 0.74$; $df = 20$; $P = 0.78$). The interaction 'amendments'× 'species' × 'exposure time' was not significant ($F = 0.40$; $df = 20$; $P = 0.99$) indicating that the effect of each factor was thus independent of the levels of the other factors.

6.4.1.1 Effect of pre-exposure of *G. rostochiensis* and *G. pallida* cysts to amendments on hatching

The hatching data obtained from each treatment were fitted to the logistic model with R^2 of at least 0.96. The final percentage hatch (parameter *c*) from cysts of *G. rostochiensis* retrieved from soil 16 weeks after incorporation of amendments varied between 61.2 and 81.3% (Table 6.2). There was a significant difference in the final percentage hatch among the treatments (F = 10.83; df = 10; $P < 0.001$) (Table 6.2).

Table 6.2. Means ± standard deviations of the parameters and R^2 values of the logistic model y = c/(1 + exp(− b × (time − m))) describing the hatching in PRD of second-stage juveniles from cysts of *Globodera rostochiensis* and *G. pallida* retrieved from non-amended soil and amended soil.

Treatment	*c*	*b*	*m*	R^2
G. rostochiensis				
Non-amended soil	78.9 ± 2.4 c	1.7 ± 0.6 a	1.1 ± 0.2 ab	0.99
Pig slurry	66.3 ± 2.6 ab	1.1 ± 0.2 a	1.8 ± 0.1 cd	0.99
Pig slurry+Romchar	61.2 ± 4.9 a	1.4 ± 0.9 a	1.5 ± 0.3 abc	0.98
Cattle slurry	64.5 ± 6.9 ab	1.0 ± 0.3 a	1.7 ± 0.1 cd	0.98
Nitrogen fertiliser	61.6 ± 8.0 a	1.2 ± 0.4 a	1.1 ± 0.3 a	0.96
Romchar 1%	79.7 ± 1.4 c	1.4 ± 0.2 a	1.3 ± 0.4 abc	0.99
Romchar 0.3%	79.4 ± 4.7 c	1.4 ± 0.8 a	1.2 ± 0.2 abc	0.99
Wood chip compost	72.4 ± 4.6 ab	1.1 ± 0.5 a	1.4 ± 0.2 abc	0.98
Wood chip compost+Romchar	73.9 ± 2.5 bc	1.1 ± 0.2 a	1.7 ± 0.1 cd	0.97
Crab shell compost	67.1 ± 5.0 ab	1.5 ± 0.5 a	1.5 ± 0.5 abc	0.99
Crab shell compost+Romchar	81.3 ± 3.1 c	1.1 ± 0.1 a	2.2 ± 0.1 d	0.99
G. pallida				
Non-amended soil	80.1 ± 2.5 c	0.8 ± 0.2 a	1.4 ± 0.2 abc	0.99
Pig slurry	60.1 ± 1.7 a	0.8 ± 0.1 a	1.7 ± 0.1 abc	0.99
Pig slurry+Romchar	63.2 ± 2.0 a	0.7 ± 0.1 a	1.6 ± 0.2 abc	0.99
Cattle slurry	66.4 ± 3.0 ab	0.6 ± 0.1 a	2.1 ± 0.2 bc	0.99
Nitrogen fertiliser	60.7 ± 2.0 a	0.8 ± 0.2 a	1.1 ± 0.4 a	0.98
Romchar 1%	76.9 ± 3.9 c	0.8 ± 0.1 a	2.7 ± 0.3 cd	0.97
Romchar 0.3%	74.5 ± 4.8 c	0.9 ± 0.1 a	2.8 ± 0.2 d	0.98
Wood chip compost	65.2 ± 2.8 a	2.8 ± 0.3 c	1.1 ± 0.1 ab	0.99
Wood chip compost+Romchar	75.9 ± 2.0 c	0.8 ± 0.1 a	2.1 ± 0.2 bc	0.98
Crab shell compost	62.4 ± 6.1 a	1.2 ± 0.2 b	1.4 ± 0.3 bc	0.99
Crab shell compost+Romchar	79.6 ± 4.9 c	0.8 ± 0.1 a	2.8 ± 0.5 d	0.98

Different letters indicate significant differences ($P < 0.05$) among the treatments for each parameter: final hatching percentage (*c*), hatching rate (*b*) and time at which 50% hatch is reached (*m*).

The final percentage hatch from cysts exposed to pig slurry alone and in combination with biochar, cattle slurry, nitrogen fertiliser, wood chip compost and crab shell compost, was lower than that from cysts exposed to non-amended soil (78.9%). No significant difference was

found in the hatching rate (*b*) among the treatments (F = 1.42; df = 10; P = 0.21). The time necessary to reach 50% of the final hatch (*m*) differed significantly among treatments (F = 8.23; df = 10; $P < 0.001$); hatching occurred more slowly from cysts retrieved from soil amended with pig slurry, cattle slurry and wood chip or crab shell compost combined with biochar than from cysts retrieved from non-amended soil (Table 6.2).

Similar to the observations with *G. rostochiensis*, exposing cysts of *G. pallida* to pig slurry, alone or in combination with biochar, nitrogen fertiliser, crab shell compost, wood chip compost or cattle slurry, resulted in fewer hatched J2 (*c*) (F = 19.70; df = 10; $P < 0.001$) (Table 6.2). Differences among the treatments were also observed in the hatching rate (*b*) (F = 68.92; df = 10; $P < 0.001$) and the time necessary for 50% hatch (*m*) (F = 20.54; df = 10; $P < 0.001$).

Significant differences were found between the two PCN species for the final hatching number (*c*) (F = 4.89; df = 1; P = 0.03), hatching rate (*b*) (F = 9.11; df = 1; P = 0.004) as well as for the time to reach 50% of the final hatch (*m*) (F = 59.60; df = 1; $P < 0.001$). There was a significant interaction 'amendment' × 'species' for hatching rate (*b*) (F = 7.56; df = 10; $P < 0.001$) and for *m* (F = 11.62; df = 10; $P < 0.001$), showing that the effect of amendments on PCN hatching was different for the two species.

6.4.1.2 Effect of pre-exposure of *G. rostochiensis* and *G. pallida* cysts to amendments on infectivity of J2

Inoculating potato tubers planted in the closed containers with hatched J2 from cysts recovered from amended and non-amended soil after 16 weeks exposure showed no significant differences in the number of J2 of those species penetrated the roots (F = 0.96; df = 10; P = 0.50 and F = 0.28; df = 10; P = 0.98, for *G. rostochiensis* and *G. pallida*, respectively) (data not shown). No significant difference was found for the main factor 'species' (F = 1.76; df = 1; P = 0.19) nor for the interaction 'amendment' × 'species' (F = 0.75; df = 10; P = 0.67).

6.4.2 Effects of amendments on the reproduction of *G. rostochiensis* and *G. pallida*

Fewer cysts of *G. rostochiensis* were extracted from 500 cm^3 soil amended with pig slurry (16 ± 3.47), wood chip compost (19.5 ± 2.38), cattle slurry (21.75 ± 2.36) and nitrogen

fertiliser (29.5 ± 5.91) than in non-amended soil (67.5 ± 7.23) (F = 23.19; df = 10; P < 0.001) (Figure 6.2).

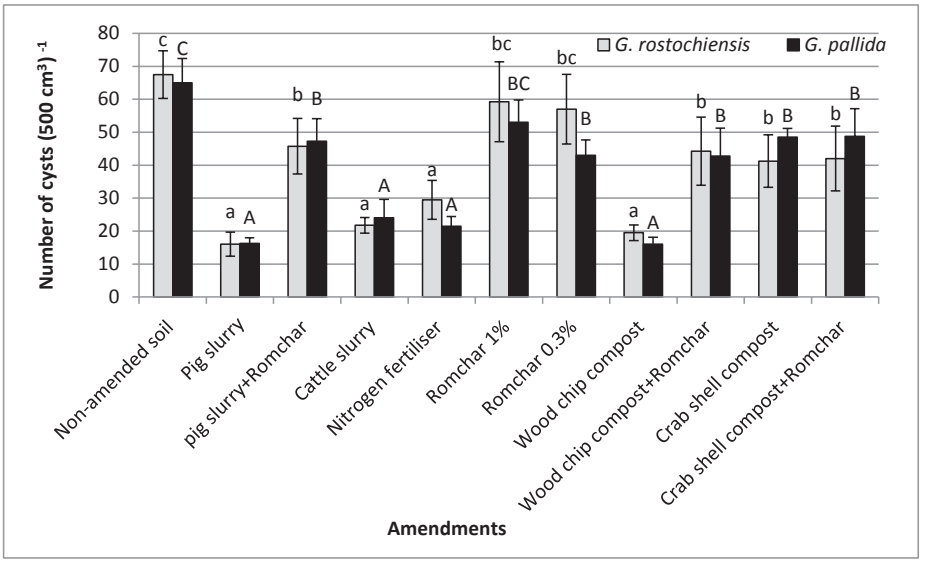


Figure 6.2. The effect of soil amendments at 170 kg N ha^{-1} on the number of cysts of *Globodera rostochiensis* and *G. pallida* per 500 cm^3 soil. Error bars represent the standard deviation of the mean. Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test (P < 0.05).

The number of eggs per cyst and the total number of eggs per cm^3 of soil were also reduced in those soils as well as in soils amended with crab shell compost alone and combined with biochar, compared with non-amended soil (F = 29.94; df = 10; P < 0.001 and F = 44.09; df = 10; P < 0.001, respectively) (Figures 6.3, 6.4). Pig slurry caused the greatest reduction in the number of *G. rostochiensis* eggs per cm^3 of soil (5.01 ± 1.07) compared with non-amended soil (39.09 ± 2.63).

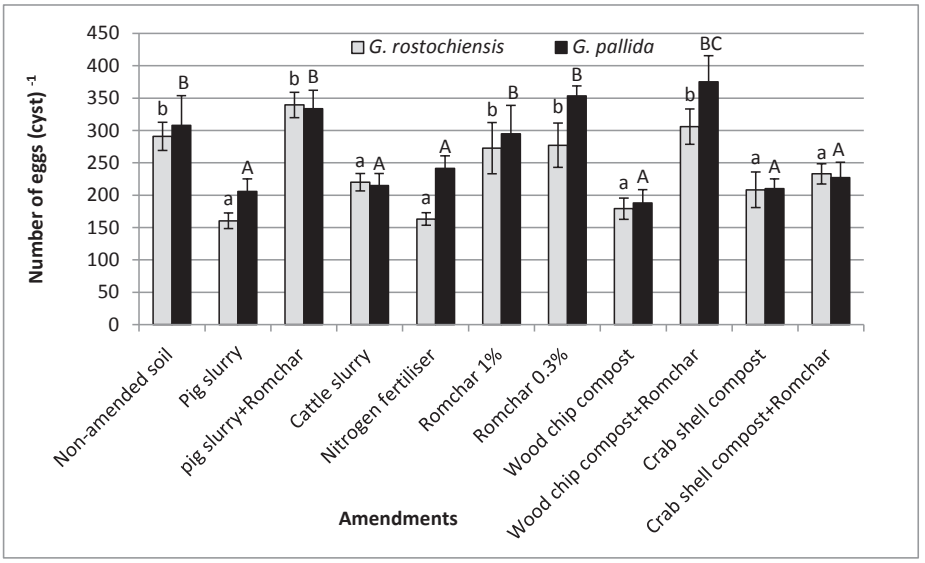


Figure 6.3. The effect of soil amendments at 170 kg N ha^{-1} on the number of eggs of *Globodera rostochiensis* and *G. pallida* per cyst. Error bars represent the standard deviation of the mean. Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

General trends of the data in the experiments with *G. pallida* were similar. Significant differences were found in the number of cysts per 500 cm^3 soil and the number of eggs per cyst among treatments ($F = 47.82$; $df = 10$; $P < 0.001$ and $F = 19.82$; $df = 10$; $P < 0.001$, respectively) (Figures 6.2-6.4). Fewer eggs per cm^3 of soil were counted in soil amended with wood chip compost (6.05 ± 1.28), pig slurry (6.67 ± 0.77), cattle slurry (10.22 ± 1.90), nitrogen fertiliser (10.42 ± 2.05), and crab shell compost alone (20.36± 1.93) and mixed with biochar (22.35 ± 5.93), compared with non-amended soil (40.54 ± 6.12) ($F = 48.37$; $df = 10$; $P < 0.001$) (Figures 6.2-6.4). However, biochar 0.3% and 1%, and both pig slurry and wood chip compost mixed with biochar had no effect on the reproduction of both PCN species. Combining biochar with pig slurry and wood chip compost resulted in fewer newly formed cysts compared to non-amended soil (Figure 6.2), but had no impact on the final egg density in the soil (Figure 6.4). Pig slurry and wood chip compost alone caused a higher reduction in reproduction of both PCN species compared with their combination with biochar.

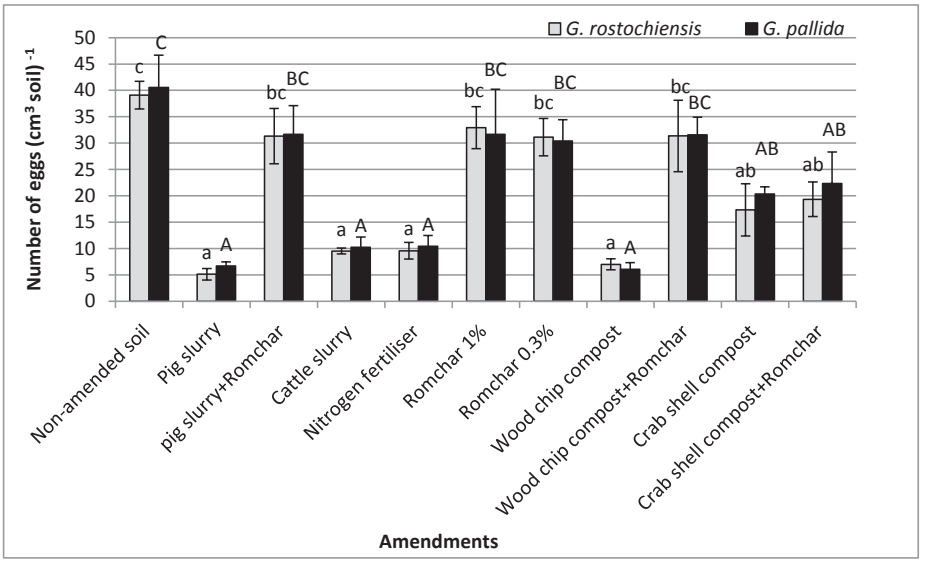


Figure 6.4. The effect of soil amendments at 170 kg N ha^{-1} on the number of eggs of *Globodera rostochiensis* and *G. pallida* per cm^3 soil. Error bars represent the standard deviation of the mean. Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

The effects of the amendments (number of cysts, number of eggs per cyst and number of eggs per cm^3 soil) were similar for *G. rostochiensis* and for *G. pallida* (Figures 6.2-6.4). A two-way-factorial ANOVA revealed that there were no significant differences in the soil populations densities (eggs per cm^3 soil) for the main factor 'species' ($F = 1.18$; $df = 1$; $P = 0.28$) nor for the interaction 'soil amendments' × 'species' ($F = 1.18$; $df = 10$; $P = 0.32$).

6.4.2.1 Effects of amendments on hatching of *G. rostochiensis* and *G. pallida*

When untreated cysts of *G. rostochiensis* were incubated in PRD collected from the different soils, the final hatching percentages (parameter *c*) differed significantly among the treatments ($F = 16.33$; $df = 10$; $P < 0.001$) (Table 6.3). The final percentage hatch of *G. rostochiensis* in PRD collected from most amended soils (74-82%) was significantly lower than that in PRD from non-amended soil (91%). The exceptions were PRD from soils amended with two concentrations of biochar for which no significant differences in the total percentage hatch (88%) were observed in comparison with the control. Significant differences were found in the hatching rate (*b*) among the treatments ($F = 11.15$; $df = 10$; $P < 0.001$); the time necessary for

50% hatch (*m*) also differed among treatments ($F = 83.10$; $df = 10$; $P < 0.001$). Hatch from cysts exposed to PRD from soil amended with wood chip compost was faster (1.4 weeks to reach 50% hatch) than that from cysts exposed to PRD from other amended soils and control. Hatch from cysts exposed to PRD from soil amended with biochar 1% and 0.3%, and the combination of biochar and wood chip compost was delayed and reached 50% (*m*) in 4.4, 4.1 and 4.0 weeks, respectively (Table 6.3).

Table 6.3. Means ± standard deviations of the parameters and R^2 values of the logistic model $y = c/(1 + \exp(-b \times (\text{time} - m)))$ describing the hatching in PRD of second-stage juveniles from cysts of *Globodera rostochiensis* and *G. pallida* exposed to potato root diffusate collected from non-amended soil and amended soil.

Treatment	*c*	*b*	*m*	R^2
G. rostochiensis				
Non-amended soil	90.8 ± 3.4 d	0.7 ± 0.1 abcd	2.9 ± 0.1 d	0.99
Pig slurry	71.2 ± 1.5 a	1.0 ± 0.1 def	2.0 ± 0.3 b	0.99
Pig slurry+Romchar	81.0 ± 3.6 bc	0.7 ± 0.3 abc	2.9 ± 0.4 d	0.99
Nitrogen fertiliser	71.3 ± 1.8 a	1.2 ± 0.5 f	2.1 ± 0.1 bc	0.99
Cattle slurry	74.1 ± 1.7 ab	1.0 ± 0.4 def	2.1 ± 0.2 b	0.99
Romchar 1%	88.0 ± 7.9 cd	0.5 ± 0.1 a	4.4 ± 0.5 e	0.99
Romchar 0.3%	88.2 ± 5.1 cd	0.6 ± 0.1 ab	4.1 ± 0.4 e	0.99
Wood chip compost	73.9 ± 3.3 ab	1.0 ± 0.2 cdef	1.4 ± 0.1 a	0.99
Wood chip compost+Romchar	80.1 ± 3.1 bc	1.0 ± 0.1 cdef	4.0 ± 0.2 e	0.99
Crab shell compost	81.7 ± 2.2 bc	0.8 ± 0.1 bcde	2.5 ± 0.1 cd	0.99
Crab shell compost+Romchar	81.7 ± 2.3 bc	0.8 ± 0.1 bcde	2.9 ± 0.2 d	0.99
G. pallida				
Non-amended soil	88.9 ± 1.5 d	0.7 ± 0.1 abc	4.1 ± 0.1 cd	0.99
Pig slurry	70.6 ± 6.2 a	0.8 ± 0.1 bc	3.6 ± 0.2 bc	0.99
Pig slurry+Romchar	83.1 ± 7.3 cd	0.7 ± 0.1 abc	4.7 ± 0.4 de	0.99
Cattle slurry	74.3 ± 2.6 abc	0.8 ± 0.1 bc	3.5 ± 0.3 bc	0.99
Nitrogen fertiliser	72.4 ± 2.3 ab	0.9 ± 0.2 c	3.4 ± 0.1 bc	0.99
Romchar 1%	82.9 ± 4.4 cd	0.6 ± 0.1 ab	4.4 ± 0.2 de	0.99
Romchar 0.3%	85.8 ± 4.6 d	0.5 ± 0.1 a	4.8 ± 0.5 de	0.99
Wood chip compost	74.6 ± 1.5 abc	0.6 ± 0.1 ab	2.4 ± 0.1 a	0.99
Wood chip compost+Romchar	81.2 ± 4.2 bcd	0.7 ± 0.1 abc	5.0 ± 0.3 de	0.99
Crab shell compost	73.9 ± 1.8 abc	0.8 ± 0.1 bc	3.3 ± 0.2 bc	0.98
Crab shell compost+Romchar	82.8 ± 3.5 cd	0.6 ± 0.1 ab	3.7 ± 0.4 bc	0.99

Different letters indicate significant differences ($P < 0.05$) among the treatments for each parameter: final hatching percentage (*c*), hatching rate (*b*) and time at which 50% hatch is reached (*m*).

Similarly, for cyst of *G. pallida*, differences were found between the final percentage hatch in PRD from different amended soils ($F = 8.99$; $df = 10$; $P < 0.001$): final hatch of *G. pallida* was lower in PRD collected from soil amended with pig slurry, nitrogen fertiliser, cattle slurry, crab shell and wood chip compost than from PRD from non-amended soil. No significant

differences were found in the hatching rate (*b*) (F = 5.03; df = 10; P = 0.06). However, significant differences were found in the time necessary for 50% hatch (*m*) between treatments (F = 31.82; df = 10; $P < 0.001$) (Table 6.3). Similar to *G. rostochiensis*, juveniles of *G. pallida* hatch faster in PRD from soil amended with wood chip compost (2.4 weeks to reach 50% hatch) than those from other amendments and control. Unlike *G. rostochiensis*, no delay was observed in the PRD from amended soil with biochar 0.3% and 1%.

Significant differences were found between the two PCN species for the parameters *b* (F = 29.93; df = 1; $P < 0.001$) and *m* (F = 456.22; df = 1; $P < 0.001$). However, parameter *c* showed no differences between species (F = 1.61; df = 1; P = 0.21). The interactions 'amendment' × 'species' for the three parameters was significant ($P < 0.001$).

6.4.2.2 Effects of amendments on migration of J2 of *G. rostochiensis* and *G. pallida*

The lowest percentage of J2 of *G. rostochiensis* migrating through the sand columns was observed in those soaked with PRD collected from soil amended with pig slurry (15%), wood chip compost (15%), nitrogen fertiliser (16%) and cattle slurry (17%) (F = 83.78; df = 11; $P <$ 0.001) (Figure 6.5). The same was observed for J2 of *G. pallida* (F = 42.97; df = 11; $P < 0.001$) where about 20% of J2 were able to migrate in soils with these solutions. The highest migration of J2 of *G. rostochiensis* and *G. pallida* occurred in sand columns with tap water, PRD collected from non-amended soil, biochar 0.3% and 1% (all around 65%) (Figure 6.5). Intermediate numbers of J2 migrated in soil amended with crab shell compost (40%) and when biochar was added to pig slurry (35%), wood chip (55%) and crab shell compost (40%) (Figure 6.5).

The number of juveniles that migrated through the sand column was similar for both PCN species (F = 19.54; df = 1; P = 0.06) and this was not influenced by the type of soil amendment (no interaction 'soil amendments' × 'species', F = 4.27; df = 11; P = 0.09).

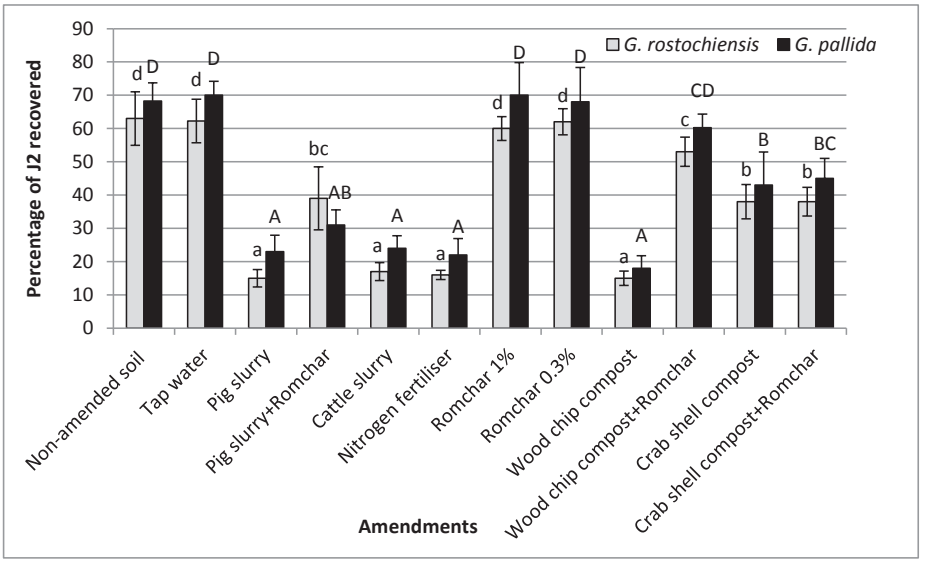


Figure 6.5. Migration of second-stage juveniles of *Globodera rostochiensis* and *G. pallida* through sand columns soaked with PRD collected from amended and non-amended soil and tap water. Error bars represent the standard deviation of the mean. Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

6.4.2.3 Effects of amendments on infectivity of J2 of *G. rostochiensis* and *G. pallida* on potato

When exposing potato plants to cysts, significant differences were found between the numbers of J2 of *G. rostochiensis* and *G. pallida* within the roots in amended soil and those in non-amended soil ($F = 21.14$; $df = 10$; $P < 0.001$ and $F = 29.64$; $df = 10$; $P < 0.001$, respectively). Fewer juveniles of both PCN species were detected in the potato roots in the soil incorporated with all amendments (Figure 6.6 A). There were no significant differences between the number of J2 of *G. rostochiensis* and *G. pallida* that penetrated the roots ($F = 138.44$; $df = 1$; $P = 0.12$). No interaction was observed between 'soil amendments' and 'species' ($F = 1.29$; $df = 10$; $P = 0.27$).

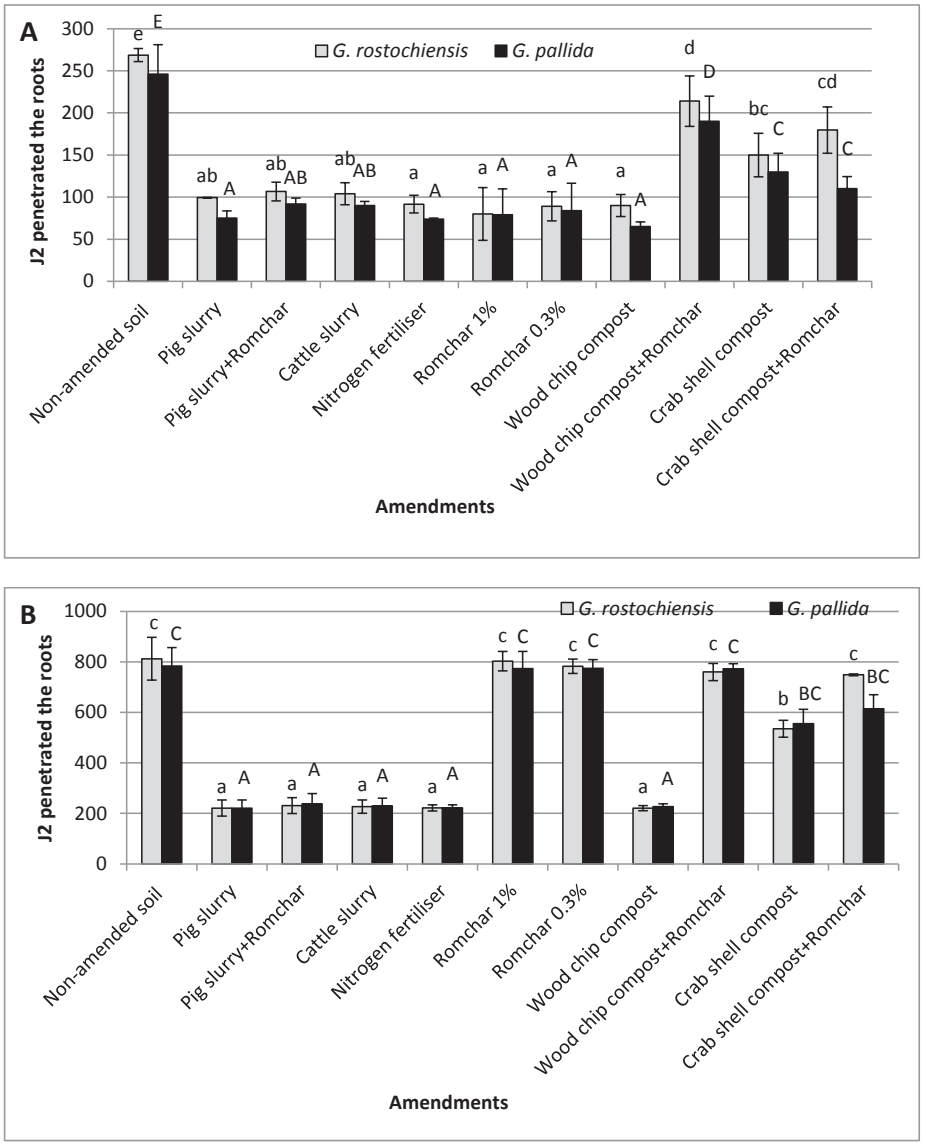


Figure 6.6. Penetration of second-stage juveniles of *Globodera rostochiensis* and *G. pallida* in the roots of potatoes planted in non-amended and amended soil and inoculated with cysts (A) or freshly hatched second-stage juveniles (B). Error bars represent the standard deviation of the mean. Bars within a nematode species followed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

The infectivity of J2 of both PCN species in amended soil was determined using freshly hatched J2 instead of cysts. Lower invasion rates of PCN were observed in potato roots from soil amended with pig slurry, wood chip compost, nitrogen fertiliser, cattle slurry, pig slurry mixed with Romchar, and crab shell compost ($F = 162.73$; $df = 10$; $P < 0.001$ for *G. rostochiensis* and $F = 105.93$; $df = 10$; $P < 0.001$ for *G. pallida*) (Figure 6.6 B). There was no effect of the other amendments on the numbers of J2 that penetrated the roots.

There was no significant differences in infectivity between *G. rostochiensis* and *G. pallida* ($F = 0.43$; $df = 1$; $P = 0.52$) or interaction 'soil amendments' and 'species' ($F = 0.75$; $df = 10$; $P = 0.68$).

6.4.2.4 Chemical analysis of soil amendments

As variable amounts of C were supplied with the amendments, the changes in C content in the soil were amendment-specific (Table 6.4). The increase in C content was highest for 1% Romchar, while no increase was observed for the mineral N fertiliser. The chemical analysis of the soil samples taken one week after the incorporation of amendments showed an increase in the amount of ammonium (NH_4-N) in the soil amended with nitrogen fertiliser (ammonium nitrate) (122.97 mg kg^{-1}soil), pig slurry (22.90 mg kg^{-1}soil) and cattle slurry (24.43 mg kg^{-1}soil) ($F = 25.58$; $df = 10$; $P < 0.001$) caused by the addition of these amendments compared with that in non-amended soil (4.0 mg kg^{-1}soil). Higher mineral nitrogen contents (NH_4-N and NO_3-N) were also measured in the soils amended with nitrogen fertiliser (151.33 mg kg^{-1}soil), pig slurry (75.24 mg kg^{-1}soil), cattle slurry (77.14 mg kg^{-1}soil) and pig slurry mixed with Romchar (59.28 mg kg^{-1}soil) and to a lesser extent in soil amended with crab shell compost alone (22.65 mg kg^{-1}soil) and in combination with Romchar (22.48 mg kg^{-1}soil), compared with other amendments and non-amended soil (12.95 mg kg^{-1}soil). The results of the soil analysis at the end of the experiment (16 weeks after amending soil with different materials) revealed a higher ammonium content of soil amended with pig slurry (5.21 mg kg^{-1}soil) and cattle slurry (5.30 mg kg^{-1}soil) compared with that of non-amended soil (3.43 mg kg^{-1}soil) ($F = 3.69$; $df = 10$; $P < 0.001$) (Table 6.4).

Table 6.4. Chemical soil properties in pots with and without amendments, measured at the end of the experiment (16 weeks after incorporation). Values are the means of 4 replicates.

Treatment	pH-KCl	EC µS cm^{-1}	OC %	Total N %	C:N	NH_4-N (mg kg^{-1})	Mineral N (mg kg^{-1})	P-AL (mg kg^{-1})	Ca-AL (mg kg^{-1})	K-AL (mg kg^{-1})	Mg-AL (mg kg^{-1})
Non-amended soil	5.9 a	104.3 a	0.9 a	0.1 a	9.0 a	3.4 ab	9.1 ab	238.8 ab	805.2 a	238.8 ab	121.3 bc
Pig slurry	6.5 dc	115.5 b	1.1 ab	0.1 a	11 ab	5.2 c	15.2 c	252.8 b	867.3 a	363.5 d	158.0 ef
Pig slurry+Romchar	6.3 cd	116.5 b	1.3 b	0.1 a	13 bc	4.5 abc	11.3 abc	244.0 ab	854.8 a	266.5 abc	143.5 de
Cattle slurry	6.3 bcd	123.5 b	1.2 b	0.1 a	12 b	5.3 c	12.7 c	241.3 ab	875.8 a	306.6 cd	152.0 def
Nitrogen fertiliser	6.0 ab	103.5 a	0.9 a	0.1 a	9.0 a	3.7 abc	7.9 a	228.1 a	797.8 a	238.1 a	119.5 ab
Romchar 1%	6.4 cde	111.5 a	1.5 c	0.1 a	15.0 d	3.4 ab	8.6 ab	229.5 a	880.1 a	258.3 abc	136.8 bcd
Romchar 0.3%	6.1 abc	97.8 a	1.2 b	0.1 a	12.0 b	3.3 a	9.0 ab	231.5 a	838.0 a	247.1 abc	125.5 abc
Wood chip compost	6.5 e	114.5 b	1.1 ab	0.1 a	11.0 ab	4.2 abc	8.6 ab	239.5 ab	946.2 a	305.5 cd	136.8 bcd
Wood chip compost+Romchar	6.2 abc	99.8 a	1.3 b	0.1 a	13.0 bc	3.7 abc	7.8 a	237.8 ab	874.0 a	277.6 abc	139.8 cd
Crab shell compost	7.4 f	156.3 c	1.3 b	0.1 a	13.0 bc	3.8 abc	8.3 ab	415.3 d	2364.0 c	246.5 abc	181.7 g
Crab shell compost+Romchar	7.22 f	145.3 c	1.6 b	0.1 a	16.0 d	3.70 abc	7.6 a	363.8 c	1835.3 b	241.7 abc	164.5 f

EC = Electrical conductivity; OC= organic carbon; Total N = total nitrogen; C:N = Carbon:nitrogen ratio; NH_4-N = ammonium; P-AL = Ammonium lactate extractable phosphorous; Ca-AL = Ammonium lactate extractable calcium; K-AL = Ammonium lactate extractable potassium; Mg-AL = Ammonium lactate extractable magnesium. Different letters in each column indicate significant differences ($P < 0.05$) among the treatments for each parameter.

The soil pH increased after incorporation of the amendments over a period of 16 weeks ($F = 43.70$; $df = 10$; $P < 0.001$). Compared with non-amended soil with a pH of 5.99, the highest pH (7.40) was observed in soil amended with crab shell compost alone and in combination with Romchar (7.22), followed by pig slurry (6.50) and cattle slurry (6.31). The addition of the mineral nitrogen fertiliser, Romchar 0.3% and the combination of Romchar and wood chip compost had no effect on the soil pH (Table 6.4).

Electrical conductivity (EC) was higher for the soils amended with crab shell compost (with or without Romchar), cattle slurry, pig slurry (with or without Romchar) and wood chip compost than for non-amended soil. Regarding the nutrient content of the soil, soils amended with crab shell compost alone or mixed with Romchar were an important source of P and Ca, while the applied doses of pig slurry, cattle slurry and wood chip compost supplied high amounts of K compared with the other amendments. Moreover, amending soil with pig slurry alone and mixed with biochar, cattle slurry, and crab shell compost with and without biochar resulted in higher supply of Mg in the soil (Table 6.4).

6.4.2.5 Phospholipid fatty acid (PLFA) composition of the soil microbiota

The total concentration of PLFAs differed significantly among treatments ($F = 6.76$; $df = 10$; $P < 0.001$). More PLFAs were detected in soils amended with pig slurry alone and in combination with biochar followed by cattle slurry, crab shell compost alone and mixed with biochar, and wood chip compost compared with those in non-amended soil (Figure 6.7). For all marker PLFAs of selected microbial groups (bacteria, fungi, arbuscular mycorrhizal fungi (AMF) and actinomycetes), significant differences were observed among treatments ($P < 0.001$) (Table 6.5). The highest bacterial and fungal marker PLFAs each, were found in soil amended with pig slurry alone and mixed with biochar and cattle slurry.

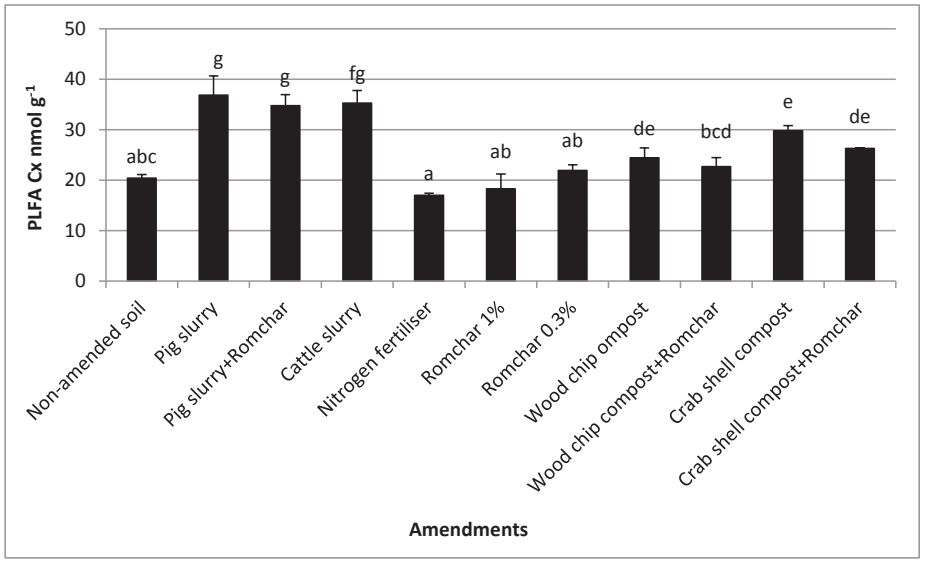


Figure 6.7. Total biomass of phospholipid fatty acids detected in amended and non-amended soils. Error bars represent the standard deviation of the mean. Bars with different letters are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

Table 6.5. Mean absolute abundance ± standard deviation of individual biomarker phospholipid fatty acid in amended and non-amended soil (n = 4).

Organism	PLFA	Non-amended soil	Pig slurry	Pig slurry+ Romchar	Cattle slurry	Nitrogen fertiliser	Romchar 1%	Romchar 0.3%	Wood chip compost	Wood chip compost+ Romchar	Crab shell compost	Crab shell compost+ Romchar
Actinomycetes	**10Me-C18:0**	0.60 ± 0.06	1.01 ± 0.09	1.21 ± 0.20	1.11 ± 0.08	0.50 ± 0.02	0.59 ± 0.01	0.54 ± 0.10	0.55 ± 0.02	0.55 ± 0.03	0.63 ± 0.01	0.57 ± 0.00
	10Me-C16:0	0.69 ± 0.01	1.11 ± 0.11	1.11 ± 0.13	1.11 ± 0.10	0.62 ± 0.02	0.82 ± 0.01	0.77 ± 0.04	0.83 ± 0.03	0.82 ± 0.10	1.06 ± 0.01	0.93 ± 0.01
	10Me-C17:0	0.61 ± 0.02	1.20 ± 0.20	1.26 ± 0.25	1.16 ± 0.19	0.59 ± 0.00	0.72 ± 0.09	0.66 ± 0.03	0.70 ± 0.03	0.69 ± 0.04	0.83 ± 0.02	0.71 ± 0.00
Total actinomycetes		**1.90 ± 0.05 a**	**3.32 ± 0.10 b**	**3.59 ± 0.08 b**	**3.38 ± 0.03 b**	**1.71 ± 0.07 a**	**2.13 ± 0.11 a**	**1.98 ± 0.11 a**	**2.08 ± 0.14 a**	**2.06 ± 0.14 a**	**2.51 ± 0.21 a**	**2.21 ± 0.18 a**
fungi	**C18:2n9,12**	1.03 ± 0.26	2.34 ± 0.51	2.04 ± 0.37	1.94 ± 0.26	0.73 ± 0.10	1.09 ± 0.21	1.11 ± 0.20	1.38 ± 0.31	1.16 ± 0.14	1.46 ± 0.11	1.66 ± 0.21
	C18:1c9	1.25 ± 0.16	2.50 ± 0.48	3.88 ± 1.59	3.50 ± 0.04	0.99 ± 0.03	1.29 ± 0.13	1.26 ± 0.05	1.60 ± 0.02	1.34 ± 0.10	1.76 ± 0.02	1.81 ± 0.33
AMF	**C16:1c11**	1.12 ± 0.06	2.10 ± 0.30	1.99 ± 0.28	1.55 ± 0.07	0.90 ± 0.01	1.43 ± 0.15	1.23 ± 0.07	1.58 ± 0.03	1.38 ± 0.14	1.92 ± 0.11	1.54 ± 0.01
Total fungi		**3.39 ± 0.11 a**	**6.94 ± 0.20 b**	**7.92 ± 1.08 b**	**6.99 ± 1.03 b**	**2.62 ± 0.13 a**	**3.81 ± 0.17 a**	**3.60 ± 0.08 a**	**4.56 ± 0.12 a**	**3.88 ± 0.12 a**	**5.14 ± 0.23 b**	**5.01 ± 0.13 b**
Gram negative bacteria	**C18:1c11**	2.50 ± 0.28	4.24 ± 0.74	4.27 ± 0.51	3.98 ± 0.10	2.05 ± 0.02	2.68 ± 0.32	2.72 ± 0.27	2.68 ± 0.12	2.62 ± 0.12	3.41 ± 0.00	3.11 ± 0.14
	C19:0cy	0.82 ± 0.06	1.26 ± 0.17	1.36 ± 0.20	1.20 ± 0.59	0.68 ± 0.03	0.91 ± 0.07	0.89 ± 0.04	0.86 ± 0.02	0.94 ± 0.07	1.06 ± 0.04	0.91 ± 0.03
	C16:1c9	2.01 ± 0.09	3.78 ± 0.72	3.40 ± 0.69	3.11 ± 0.35	1.69 ± 0.11	2.27 ± 0.38	2.16 ± 0.17	2.27 ± 0.29	2.06 ± 0.14	2.87 ± 0.11	2.43 ± 0.11
	C16:1t9	0.66 ± 0.01	1.10 ± 0.08	1.13 ± 0.15	1.10 ± 0.08	0.61 ± 0.04	0.73 ± 0.01	0.74 ± 0.06	0.76 ± 0.04	0.74 ± 0.05	1.02 ± 0.01	0.96 ± 0.12
	C17:0cy	0.66 ± 0.02	1.18 ± 0.09	1.21 ± 0.18	1.10 ± 0.45	0.62 ± 0.01	0.74 ± 0.00	0.76 ± 0.05	0.75 ± 0.06	0.73 ± 0.07	0.92 ± 0.01	0.78 ± 0.00
Total gram negative bacteria		**6.64 ± 0.86 a**	**11.55 ± 1.56 c**	**11.37 ± 1.46 c**	**10.49 ± 1.36 c**	**5.65 ± 0.69 a**	**7.33 ± 0.94 a**	**7.26 ± 0.92 a**	**7.32 ± 0.93 a**	**7.10 ± 0.87 a**	**9.29 ± 1.19 b**	**8.20 ± 1.06 a**
Gram positive bacteria	**a-C15:0**	1.04 ± 0.15	1.97 ± 0.24	1.68 ± 0.55	1.40 ± 0.47	0.91 ± 0.02	1.31 ± 0.08	1.15 ± 0.07	1.30 ± 0.16	1.14 ± 0.14	1.63 ± 0.08	1.34 ± 0.02
	i-C16:0	0.56 ± 0.01	1.01 ± 0.08	0.98 ± 0.20	1.00 ± 0.11	0.49 ± 0.02	0.63 ± 0.00	0.61 ± 0.03	0.67 ± 0.03	0.63 ± 0.05	0.85 ± 0.02	0.70 ± 0.02
	i-C17:0	0.76 ± 0.01	1.39 ± 0.27	1.33 ± 0.21	1.50 ± 0.48	0.60 ± 0.04	0.91 ± 0.09	0.82 ± 0.07	0.92 ± 0.05	0.88 ± 0.08	1.10 ± 0.05	0.94 ± 0.01

Table 6.5. Continued.

Organism	PLFA	Non-amended soil	Pig slurry	Pig slurry+Romchar	Cattle slurry	Nitrogen fertiliser	Romchar 1%	Romchar 0.3%	Wood chip compost	Wood chip compost+ Romchar	Crab shell compost	Crab shell compost+ Romchar
	i-C15:0	1.50 ± 0.22	3.09 ± 0.42	2.47 ± 0.92	2.50 ± 0.17	1.29 ± 0.02	1.91 ± 0.12	1.65 ± 0.09	2.07 ± 0.26	1.72 ± 0.21	2.55 ± 0.19	1.97 ± 0.14
Total gram positive bacteria		**3.85 ± 0.41 a**	**7.46 ± 0.91 b**	**6.46 ± 0.64 b**	**6.40 ± 0.64 b**	**3.29 ± 0.36 a**	**4.76 ± 0.56 a**	**4.22 ± 0.45 a**	**4.96 ± 0.61 a**	**4.37 ± 0.47 a**	**6.13 ± 0.75 b**	**4.95 ± 0.55 a**
non-specific bacteria	**C15:0**	0.14 ± 0.00	0.28 ± 0.04	0.23 ± 0.07	0.19 ± 0.32	0.12 ± 0.00	0.08 ± 0.08	0.14 ± 0.02	0.17 ± 0.02	0.15 ± 0.01	0.20 ± 0.01	0.17 ± 0.01
	C14:0	0.24 ± 0.03	0.47 ± 0.06	0.32 ± 0.19	0.35 ± 0.61	0.18 ± 0.01	0.28 ± 0.00	0.24 ± 0.01	0.31 ± 0.04	0.25 ± 0.03	0.35 ± 0.03	0.27 ± 0.04
	C16:0	3.54 ± 0.03	6.18 ± 0.62	5.53 ± 0.92	5.20 ± 0.12	3.02 ± 0.27	3.94 ± 0.20	3.99 ± 0.20	4.30 ± 0.58	4.13 ± 0.29	5.27 ± 0.45	4.67 ± 0.14
	C17:0	0.14 ± 0.03	0.24 ± 0.04	0.26 ± 0.02	0.26 ± 0.11	0.11 ± 0.00	0.12 ± 0.01	0.15 ± 0.02	0.14 ± 0.00	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.00
	C18:0	0.77 ± 0.17	1.17 ± 0.14	1.28 ± 0.02	1.50 ± 0.01	0.55 ± 0.02	0.69 ± 0.02	0.76 ± 0.03	0.72 ± 0.02	0.78 ± 0.00	0.85 ± 0.08	0.78 ± 0.00
Total bacteria		**15.30 ± 0.98 a**	**27.34 ± 1.74 c**	**25.45 ± 1.57 c**	**24.39 ± 1.46 c**	**12.92 ± 0.83 a**	**17.20 ± 1.10 a**	**16.76 ± 1.09 a**	**17.92 ± 1.17 a**	**16.93 ± 1.10 a**	**22.27 ± 1.45 b**	**19.19 ± 1.28 b**

AMF = arbuscular mycorrhizal fungi. Different letters in each row indicate significant differences ($P < 0.05$) among the treatments.

6.5 Discussion

The present study was carried out to investigate the effects of several amendments from different origins *i*) on the viability of both PCN species in the absence of a host crop, simulating field conditions (crop rotation), *ii*) on the reproduction of PCN on potato and *iii*) to determine the possible modes of action of nematode suppression by amendments. The viability of cysts content of both PCN species was reduced in soil amended with pig slurry, cattle slurry, nitrogen fertiliser, crab shell compost, pig slurry mixed with biochar and wood chip compost in the absence of a host. This shows that the application of these organic materials and artificial nitrogen fertiliser in crops rotated with potato lowers the PCN infestation. The percentage mortality of encysted eggs due to aforementioned amendments varied between 15 and 22.5% for *G. rostochiensis* and between 18 and 25% for *G. pallida*. These relatively low levels of mortality caused by amendments could be attributed to the presence of physical barriers, *i.e*., the cyst wall and the egg shell which protect the unhatched J2 from the toxic compounds released during the degradation of amendments. However, it should be noted that 22-25% mortality caused by amendments occurred within a period of 16 weeks while considering crop rotations of one in three years potato, cysts will remain longer in the fields. This suggests that this percentage of mortality in amended soils in the absence of potato is comparable with the PCN average natural decline of 10-40% per year.

The exposure of cysts to amendments for sixteen weeks did not affect the infectivity of juveniles hatched from these cysts. This suggests the direct effect of amendments on the eggs inside the cysts by killing them in the absence of a host with no effect on the infectivity of J2.

Besides the direct effect of the amendments on the viability of PCN in the absence of a host crop, amending soil with pig slurry, wood chip compost, cattle slurry, nitrogen fertiliser, and crab shell compost alone and blended with biochar, resulted in significantly less reproduction of both PCN species during the potato development. The greatest reduction in the reproduction of both PCN species was achieved in the soil amended with pig slurry and wood chip compost (87% and 82%, respectively). Renčo *et al*. (2007) also showed that five composts originating from plants, animals or fungi, at all concentrations reduced the number of eggs and juveniles of *G. rostochiensis* by 78%.

Application of biochar alone and in combination with wood chip compost as well as with pig slurry had no inhibitory effect on the reproduction of both PCN species. Few studies have investigated the effects of biochar addition on plant-parasitic nematodes. Zhang *et al*. (2013) reported a decrease in the population of several nematodes, *e.g*., *Hirschmanniella* and *Pratylenchus* in wheat fields amended with biochar. My findings were consistent with those of Perry and Beane (1988) who reported no suppressive effect of activated charcoal on the final population density of *G. rostochiensis* in soil amended with charcoal. However, there have been some reports of the promising effects of biochar on several plant pathogens and pest. Soil-applied biochar induced systemic resistance to the foliar fungal pathogens *Botrytis cinerea* (gray mold) and *Leveillula taurica* (powdery mildew) on pepper and tomato and to the broad mite pest (*Polyphagotarsonemus latus*) on pepper (Elad *et al*., 2010). Wood biochar and greenhouse waste biochar suppressed the incidence of *B. cinerea*, *Colletotrichum acutatum* and *Podosphaera apahanis* in strawberry plants through stimulation of defense pathways in plants (Harel *et al*., 2012). It is interesting to note that in these experiments, biochar reduced the suppressive effect of pig slurry and wood chip compost on PCN reproduction. This might be attributed to the absorbance of suppressive compounds by the high surface area of biochar, *e.g*., ammonium or disulphide compounds present in these amendments or produced during their decomposition.

To unravel mechanisms of nematode suppression, soil chemical and biological analyses were performed. The results of the chemical analysis of the samples indicated that the addition of nitrogen fertiliser (ammonium nitrate), pig slurry and cattle slurry resulted in a significant increase in the amount of ammonium in the soil. Previous studies attributed the suppressive effect of animal manures on nematodes to high ammonium concentrations and volatile fatty acids (Rodríguez-Kabana *et al*., 1981; Rodríguez-Kabana, 1986; Chitwood, 2002; Oka, 2010). It should be noted that the nematicidal activity of ammonia and ammonium in the soil is known to be short-lived (a few days to weeks) (Tenuta & Lazarovits, 2002). Therefore, less influence of amendments on the reproduction of *G. pallida* was expected due to its hatching over much longer periods of time when the toxic ammonium would not be present in the soil. However, these amendments affected the reproduction of this species in a same manner to that of *G. rostochiensis*. Hence, the effect of other factors including pH, volatile fatty acids and the changes in microbial community of the soil should also be considered.

The crab shell compost amendment also caused a significant reduction in the reproduction of PCN. These results are in accordance with previous studies on the role of chitinous amendments on nematodes (Mian *et al*. 1982; Spiegel *et al*., 1987; De Jin *et al*., 2005). Chitin amendments have been shown to suppress nematode populations by the release of ammonia during their decomposition and by the stimulation of chitinolytic organisms that parasitize nematode eggshells (Spiegel *et al*., 1987, 1988). However, the results of the soil analysis showed an increase in total mineral nitrogen but no significant amount of ammonium in the crab shell amended soil. Therefore, the effect of ammonium seems to be unlikely. The increase in mineral nitrogen could be explained by the conversion of ammonium nitrogen to nitrate nitrogen during the 8 months incubation of soil amended with crab shell compost. The crab shell compost was incorporated into the soil 8 months before use to stimulate the chitinolytic organisms to increase their populations to levels adequate for effective nematode control (Rodríguez-Kabana *et al*., 1987). The suppressive effect of wood chip compost was also not associated with the generation of ammonium because no significant amount of ammonium was detected in wood chip amended soil.

To better understand the mechanisms of nematode suppression in amended soils, the changes in the microbial community structures and the microbial biomass were monitored using PLFA analysis. The results confirmed an increase in the total biomass of the microbial community as a result of amending soil with organic materials. Particularly, the reduction in viability and reproduction of PCN in soil amended with pig and cattle slurries, crab shell compost and wood chip compost can be partially associated with the enhancement of microbial biomass in such organic soils. The PLFA analysis was not sufficiently discriminating to identify populations of microorganisms that might be involved in the reduction of PCN viability and reproduction. It has been shown that soils with a high biological diversity and activity, such as natural or organically-amended agricultural soils are more suppressive to soil-borne diseases than conventionally-managed ones (van Bruggen, 1995; Mader *et al*., 2002). Some of the introduced or shifted organisms contribute to the suppressive activity of the amended soils through any of four principal mechanisms of biological control: competition, antibiosis, parasitism/predation, and induced systemic resistance (Lockwood, 1988). Hallmann *et al*. (1999) suggested that bacterial species, especially endophytes, which were eliminated or specifically promoted by the chitin amendment, might contribute to the observed suppressiveness of

M. incognita. Hoitink *et al*. (1997) also suggested that the colonisation of compost by beneficial microorganisms during composting appears to be responsible for the suppression of soil-borne pathogens.

Moreover, the wood chip compost used in these experiments was prepared from agro-industrial wastes including wood chip from poplar and leek residue. This amendment yielded promising results in the management of both PCN species. It is also possible that volatile fatty acids and phenolic compounds present in composts caused reduction in both PCN species reproduction. However, the presence of these compounds was not investigated in this research. This nematode suppression may also be attributed to the presence of leek waste in the compost used in this study. It was shown that leek decomposition by microorganisms in soil leads to generation of toxic sulphur-containing compounds among which the disulphides are the most effective ones because of their high persistence, stability and slow degradation in soil (Arnault *et al*., 2004).

To investigate the life stages of PCN at which the nematode development was hampered by amendments, hatching behaviour, movement, and the infectivity of hatched J2 was studied. Cysts of each PCN species were exposed to PRD collected from soil amended with different additive materials. The hatching test revealed that there was a significant reduction in the final percentage hatch in PRD from all amended soils, except for soil amended with biochar alone. The present work indicates the inhibitory effect of these amendments on the hatching of J2 of both PCN species. Amendments might have inhibited the function of PRD and influenced its stimulatory effect by modifying the hatching factors present in PRD. These observations agree with an earlier study by Xiao *et al*. (2008) that showed reduction of *H. glycines* hatch and viability by swine manure. The reduction of PCN final percentage hatch and the delay in hatch might result in less damage to potato plants. When juveniles emerge in the soil, plants might have developed a larger root system and are more tolerant of nematode attack.

The exposure of untreated PCN cysts to PRD collected from soil amended with wood chip compost stood out as it was the only amendment that stimulated more hatching of *G. rostochiensis* and *G. pallida*. However, the multiplication of these species was significantly less than in non-amended soil, suggesting an adverse effect of wood chip compost on the hatched juveniles in the soil. Danquah *et al*. (2011) reported that garlic extracts caused 50% J2 mortality

at 983.0 µl L^{-1} concentration. They demonstrated the stimulatory effect of the garlic extracts on the hatching of *G. pallida*. The mechanisms of nematode suppression in soil incorporated with wood chip compost with leek residue are not very clear. Little is known about the effect of leek residue on nematodes. So far, the results of PLFA analysis showed that changes in microbial community could be the most reliable indicators of the nematode suppression.

The infectivity assay with the hatched juveniles gave results that differed from cysts. The results from the infectivity test with cysts showed a reduction in the number of J2 which penetrated the roots in all amended soils in comparison with the control. This could be due to the delay in hatch in soils amended with biochar alone or in combination with other amendments (wood chip compost, pig slurry and crab shell compost). This is in agreement with findings of Perry and Beane (1988) who demonstrated a delay in hatching of *G. rostochiensis*, when potatoes were planted in soil amended with the activated charcoal. They attributed the delay in the emergence of PCN J2 to the possible absorbance of hatching factors of PRD by the activated charcoal. However, no significant effect of charcoal was observed on the final population density of this species at the end of the experiment. The results obtained from the reproduction of PCN at harvest also showed that although biochar significantly delayed hatching of both PCN species, it did not reduce the final number of juveniles which hatched and penetrated the roots during the growing season.

Nevertheless, when plants were inoculated with hatched J2, fewer juveniles were detected only in the potato roots in soil amended with pig slurry, cattle slurry, nitrogen fertiliser, wood chip compost and crab shell compost. The bioassay with the sand column also showed an inhibitory effect of these amendments on the movement of J2, confirming the adverse effect of these materials on hatched juveniles. The addition of the amendments and the consequent changes in potato plant nutrition may have influenced the root system and the nematode attraction towards the roots (Sudirman & Webster, 1995). Scott and Martin (1962) showed that treatments with different ions significantly affected the electrical potential around the root tip area where J2 penetrated. Therefore, the increased concentration of ammonium ions in soil amended with nitrogen fertiliser and pig and cattle slurries and the metabolites produced by microorganisms in soil amended with these amendments and wood chip and crab shell compost may have influenced penetration of the root through diminished attractiveness of the root tips.

The microorganisms might also have affected the PRD chemicals which are responsible for nematode attraction towards roots.

The results of the experiment of the effect of amendments on the reproduction of PCN showed that the number of eggs per cyst was reduced in soil amended with pig slurry, wood chip compost, cattle slurry, nitrogen fertiliser, and crab shell compost. This suggests that these amendments changed the physiology of the root system resulting in failure of many J2 to induce proper feeding sites in the roots. By contrast, Thoden *et al*. (2011) stated that nutrient-enriched root systems could improve nematode fecundity. Amendments can enhance the plant growth and tolerance to nematodes. However, in this study, the growth parameters of potato plants were not measured. Therefore, further research is needed to better elucidate effects of application of amendments on soil-plant-nematode systems.

In summary, the release of ammonium and changes in soil microbial community can be accounted for the nematode suppression in soil amended with pig and cattle slurries. However, in this study, the suppressing effect of wood chip and crab shell compost can only be explained by the changes in soil microbiota as no significant amount of ammonium was detected in soil amended with these materials. The reduction in viability and reproduction in soil amended with mineral nitrogen fertiliser appeared to be related to the production of ammonium. Ammonium and microorganisms might have affected PCN directly by killing the eggs and juveniles or indirectly by changing the physiology of the root which may have resulted in reduced hatch and J2 movement, penetration of the roots and females' fecundity.

In conclusion, amending soil reduced the viability and reproduction of both PCN species. Animal manures (pig and cattle slurry) provided an effective and inexpensive suppression of PCN species at rates practical under agricultural practice. The long-term application of amendments may improve soil chemical, physical and biological quality. Soil nutrients (K-AL, Mg-AL and Ca-AL), pH and EC were enhanced in amended soils which could have led to improved plant growth and tolerance to nematodes. Furthermore, amendments increased the total microbial biomass of the soil. Therefore, their long-lasting application can have beneficial effects on soil biological functions. However, more attention should be paid to the application rates because the repeated addition of organic amendments and inorganic fertilisers to soil at high

rates can be a source of environmental pollution and may result in accumulation of soil P and nitrate leaching.

Chapter 7

Agricultural waste amendments improve the suppressive effect of inundation on the survival of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) in waste soil

Ebrahimi, N., Viaene, N., Aerts, J., Debode, J. & Moens, M. (2015). Agricultural waste amendments improve the suppressive effect of inundation on the survival of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) in waste soil. *European Journal of Plant Pathology* (submitted).

7.1 Introduction

Potato cyst nematodes (PCN) are passively spread in many ways including by soil adhering to potato tubers or other root crops, and packaging materials. However, the major means of distribution of PCN are both farm machinery moving PCN infested soil between fields, and soil carried along with potato tubers at harvest (Goeminne *et al*., 2011). Although harvesting methods have been developed to reduce the amount of soil adhering to tubers (Vermeulen *et al*., 2003), still on average, 2.1 Mg soil ha^{-1} is taken from the field when harvesting potato (Ruysschaert *et al*., 2004). This tare soil, together with grading and sieving soil, collected after harvesting, should be considered as a waste product and its disposal should be done in a safe manner to have no risk of PCN contamination (Anon., 2007). The potential of some physical and chemical methods to disinfest waste soil, *i.e*., steaming or heating (van Loenen *et al.*, 2003), soil fumigation and inundation (van Overbeek *et al.*, 2014), has been studied. Soil steaming is effective but requires energy and labour cost. Soil fumigation suppresses nematodes in waste soils; however, most fumigants have been phased out because of increasing concerns for human health and the environment.

Inundation is effective for the suppression of soil-borne pathogens (Strandberg, 1987; Spaull *et al*., 1992; van Overbeek *et al.*, 2014). This method effectively controlled root-knot nematodes (Duncan, 1991), *Ditylenchus dipsaci* (Muller & Van Aartrijk, 1988) and *Radopholus similis* (Stover, 1979). Spaull *et al*. (1992) showed that viability of cyst content of *G. pallida* was significantly reduced (97.7-99.7%) in flooded peat soil, with and without amendments of starch and calcium sulphate. Inundation involves the induction of anaerobic conditions in the soil (Inglett *et al*., 2005). Important chemical changes in flooded soils are denitrification (reduction of nitrate by microorganisms to molecular nitrogen (N_2)), accumulation of ammonia, reduction of sulphates, production of organic acids, methane and hydrogen sulphide, and reduction of the redox potential and soil pH (Inglett *et al*., 2005). Nematode suppression in inundated anaerobic conditions might be attributed to depletion of oxygen and generation of toxic products during anaerobic decomposition, *e.g*., organic acids and volatile compounds (Runia *et al*., 2014; van Overbeek *et al.*, 2014). The survival of nematodes may further be influenced by changes in the composition of the microbial community and the resulting production of toxic compounds (Strandberg, 1987; Momma *et al*., 2006; Momma, 2008; Viaene *et al*., 2013).

Previous studies on the effect of flooding soil on the viability of PCN eggs showed that the efficacy of inundation, and the generation of gases and toxic products in inundated conditions, depend on the temperature, the duration of inundation, soil type and soil organic content (Spaull *et al*., 1992; Runia *et al*., 2012, van Overbeek *et al.*, 2014). Spaull *et al*. (1992) showed that flooding soil for 14 weeks at 20°C caused 98% reduction in viability, while at 10°C similar effect was achieved after 50 weeks. Recently, different nematode management strategies, including organic soil amendments, solarisation, and flooding have been combined as an ecological alternative to chemicals and termed Anaerobic Soil Disinfestation (ASD) (Momma *et al*., 2006; Lamers *et al*., 2010; Butler *et al*., 2012). This strategy involves the incorporation of readily available carbon sources, irrigation to saturate the soil, and finally, covering the soil with a plastic tarp or compacting the soil. These procedures stimulate rapid growth of aerobic microorganisms resulting in increased microbial respiration and consequently depletion of oxygen and anaerobic decomposition of the organic carbon (Messiha *et al*., 2007). The by-products of anaerobic decomposition including volatile fatty acids (VFA) are known to be toxic to many fungal pathogens and nematodes (Momma *et al*., 2006; Momma, 2008). The addition of grass or Herbie 7025 (a commercial product made of plant materials) to either inundated or non-inundated soils contaminated with *G. pallida* caused more than 99.4% reduction in viability of this species (van Overbeek *et al.*, 2014).

To accelerate the anaerobiosis process in the waste soil by increasing microbial respiration, decomposable amendments such as waste products of the processing industries were added to inundated soil. The amendments used in the experiments were waste products of agro-industrial activities, *viz.* fresh and steamed potato peels and the green leaves of leek. The Belgian potato processing industry has evolved to the biggest exporter of frozen potato products in the world, with approximately 3.8 million tonnes of potato being processed. Apart from the waste soil collected in these processing industries, a large amount of potato waste (peels and cut potatoes discarded due to size, blemishes, or generally failing to meet quality standards) is also generated during potato processing. Leek is also one of the most important vegetable crops in Flanders, Belgium. This plant is cultivated in different seasons throughout the year (Bernaert *et al*., 2012a; Vandewoestijne, 2014). It is grown for its thickened cylindrical stem (white shaft); hence, the outer green leaves are the post-harvest crop waste. In addition, white shafts and the green leaves of leek contain significant levels of phenolic and sulphur-containing compounds

(Bernaert *et al*., 2012b). Therefore, I examined whether waste soil contaminated with PCN could be disinfested with a combination of inundation and the agro-industrial waste products. Calcium sulphate was also added as it was proven to reduce the survival of the cyst content, particularly when a carbon source is available (Spaull *et al.*, 1992).

The aims of this study were *i*) to evaluate if amendments of the aforementioned waste products could accelerate the negative effects of inundation on survival of PCN in waste soil, *ii*) to investigate the mechanisms involved in nematode suppression in inundated amended soils by determining the chemical and biological changes in these soils, and *iii*) to understand the effect of these treatments on PCN by studying hatching, host finding ability and root penetration of the J2.

7.2 Materials and methods

7.2.1 Potato cyst nematode culture

Cysts of *G. rostochiensis* and *G. pallida* were obtained from stock cultures and maintained on potato cv. Désirée in under greenhouse conditions (see section 3.1). Sixteen weeks after inoculation, the cysts were extracted and stored for 4 months at 4°C to overcome the diapause before they were used in the experiments.

7.2.2 Amendments

Agro-industrial waste products used in the tests were steamed potato peels (PP) obtained from Agristo, a potato processing company (Belgium), fresh potato peels (FPP), calcium sulphate ($CaSO_4$), a mixture of steamed potato peels and calcium sulphate (PP+$CaSO_4$) and green leaves of leek (L) chopped in pieces of 1-2 cm. Amendments were prepared two hours before the experimental set up. They were mixed with the soil at rates of 25 g PP, 20 g FPP, 4 g $CaSO_4$, a mixture of 25 g PP and 4 g $CaSO_4$, and 21 g L per 1 litre soil. The amounts were calculated based on the equivalent rates of organic matter applied per hectare in the field (Thoden *et al*., 2011). In all experiments, a sandy soil (74% sand, 6.4% clay, 19.6% silt; 1.4% organic matter; pH 6.9) was used. The PCN-free soil was collected from a field and was not sterilised prior to its use. It contained plant-parasitic nematodes, *e.g*., *Helicotylenchus* spp. and *Tylenchorhynchus* spp. as well as free-living nematodes, *e.g*., *Rhabditis* spp.

7.2.3 Experiment 1: Effects of inundation alone and in combination with amendments on the viability of *G. rostochiensis*

This experiment was set up to study the effect of inundation and amendments on the survival of *G. rostochiensis* in (1) non-amended soil (S+W), or soil amended with (2) steamed potato peels (S+W+PP), (3) fresh potato peels (S+W+FPP), (4) calcium sulphate (S+W+Ca), (5) a mixture of steamed potato peels and calcium sulphate (S+W+PP+Ca), and (6) green leaves of leek (S+W+L) chopped in pieces of 1-2 cm. Airtight plastic closed containers (2 L, 10 cm diam., 23 cm height) were filled with 1.5 L of non-amended soil or soil mixed with the amendments mentioned above (Figure 7.1). Non-inundated soils without amendments in closed (soil (lid)) and open containers (soil (no lid)), were included as controls. Eight retrievable nylon mesh bags, each containing 20 cysts of *G. rostochiensis*, were buried in the middle of the soil column of each container. Treatments 1-6 received 400 ml tap water to cover the soil surface up to 4 cm. Each treatment had 4 replicates. The containers were placed, completely randomized, in an incubator at 17 ± 0.4°C over a period of 8 weeks. Weekly, one bag containing cysts was retrieved from the containers. Ten cysts were used to determine the viability of the cysts' content, by both the visual assessment (OEPP/EPPO, 2013) and the trehalose-based method (see below). The other 10 cysts were used to examine the infectivity of the juveniles that were still able to hatch (see below).

Soil pH and the concentrations of short chain fatty acids (SCFA) were measured at the end of the experiment (see below). The concentrations of gases (O_2, CO_2, N_2, CH_4, NH_3 and H_2S) were determined 1 and 2 weeks after the start of the experiment. This experiment was performed twice.

Figure 7.1. Airtight closed containers filled with 1.5 L of non-amended or amended soil with or without water. Cysts were placed in retrievable nylon bags and buried in the soil.

7.2.3.1 Viability assessment

The effect of the treatments on the viability of the cyst content was estimated by both visual assessment and the trehalose based method to compare the efficacy of these viability assessing methods with cysts that have been treated in inundated (anaerobic) conditions. For the visual determination of viability, 10 cysts of *G. rostochiensis*, recovered from each container at each observation, were crushed. The volume of the obtained egg suspension was increased to 20 ml with tap water. Suspensions were kept for 24 h at 21°C because the incubation of eggs and juveniles in water overnight improves the visualization of their morphological features. Three subsamples of 2 ml were drawn from the homogenized egg suspension. The viability of eggs and J2 was determined using a dissecting microscope (100 ×). Damaged and empty eggs with non-smooth eggshells, and shrivelled disintegrated J2 with no clear lip region or stylet, were considered dead (OEPP/EPPO, 2013).

To assess the viability using the trehalose-based method, 4 subsamples were taken from the above egg suspension. Trehalose was extracted from the eggs using a detection kit (K-TREH 01/09, Megazyme International Ireland Ltd., Wicklow, Ireland) and following the protocol described by van den Elsen *et al*. (2012). The obtained data on the trehalose content were converted to the number of viable eggs based on the standard curves constructed in chapter 5.

7.2.3.2 Infectivity and hatching test

To evaluate the ability of surviving J2 to find and penetrate host roots after inundation of waste soil, an infectivity test was conducted in 0.5-L closed plastic containers planted with potato tubers of cv. Désirée (see section 3.5). The sand was infested with 10 cysts recovered from the airtight containers. Four weeks later, roots were gently removed, washed and juveniles inside the roots were stained with acid fuchsin (Byrd *et al*., 1983) to enable counting of juveniles that had penetrated the roots (see section 3.6). At the same time, hatched J2 were extracted from the 200 g sand in each container using an automated zonal centrifuge (Hendrickx, 1995).

7.2.3.3 Chemical analysis of soil

The concentrations of O_2, CO_2, N_2, CH_4, NH_3 and H_2S of the soil atmosphere in the headspace of the airtight closed containers were determined. Gas samples were drawn from the headspace of the containers via a septum installed on the lid of containers using a gastight syringe (Hamilton 1005 SL 5 mL sample lock syringe, 22GA). The gas samples were then transferred to a Perkin-Elmer Clarus 500 gas chromatograph on which the sample was separated into its components (CH_4, CO_2, H_2S, H_2, O_2 and N_2) using two analysis channels. The gases were detected using thermal conductivity detectors (TCD). The columns' temperature was fixed at 60°C. On a first channel, the gas sample was separated using a Porapak Q column. On this column, a good separation was obtained between CH_4, CO_2 and H_2S. However, H_2, O_2 and N_2 were not separated and appeared as one single air peak. On a second channel, the gas sample was first sent through a Unibeads column on which the gases were separated based on porosity. H_2, O_2 and N_2 passed quickly through this column, while the other gases were retained longer. H_2, O_2 and N_2 were then separated on a molecular sieve column (MolSieve 13X) while the other gases were flushed from the Unibeads column.

The concentration of NH_3 in the headspace of the containers was determined using a Dräger tube 'Ammonia 5/a (CH20501)'. The ammonia concentration was determined by sucking a known amount of the gas of interest through this tube using a calibrated pump. When NH_3 is present in the gas, a yellow indicator layer changes colour to blue. The length of discoloration is a measure of the amount of NH_3 present in the gas and can be read from the graduated tube.

The concentration of SCFA, i.e. acetic acid, propionic acid, (iso) butyric acid, and (iso) valeric acid, was determined in the soil water phase extract using gas chromatography coupled to a flame ionisation detector (GC-FID), 8 weeks after the start of the experiment. The method for identification and quantification of SCFA in soil water phase extract was optimised based on an in-house developed method in an EN ISO/IEC 17025 accredited environment which was validated according to the requirements of the Commission Decision No. 2002/657/EC (Anon., 2002). This method was accredited for alcohols and SCFA quantification in silage. After pH measurement, 50 ml of the soil water phase extract was pipetted in a 12 ml-tube and centrifuged at 4°C for 5 min at 2600 g. Two ml of supernatant was added to 2.5 ml of oxalic acid (0.03 M) and 0.5 ml of internal standard iso-capric acid (10 µmol ml^{-1}). After vortexing, 1.25 ml of the mixture was analysed with a GC-3900 (Varian), coupled to an autosampler (8400) and using an EC-1000 wide bore capillary column (l = 30 m, ID = 0.53 mm, FD = 1.20 µm; Alltech). The CP-1177 split/splitless injector was kept at 220°C using a liner splitter with fused silica wool (split = 1/10), while the FID was also kept at 220°C. Gas flows were electronically regulated (EFC-11): hydrogen (30 ml min^{-1}), air (300 ml min^{-1}), helium (7.2 ml min^{-1} at 6 psi), and helium as make-up (25 ml min^{-1}). All SCFA were baseline separated using a temperature gradient (80°C for 5 min, 10°C min^{-1} to 200°C, 5 min at 200°C) resulting in a total run time of 25 min. Peak identification and quantification was done according to the requirements of the Commission Decision No. 2002/657/EC (Anon., 2002) using relative retention times and a calibration curve consisting of 5 calibration standards. Results were reported as µmol ml^{-1}.

7.2.4 Experiment 2: Mechanisms of *G. rostochiensis* and *G. pallida* suppression in inundated amended soils

The objectives of this experiment were *i*) to examine if the effect of inundation alone and in combination with amendments on *G. pallida* is similar to that on *G. rostochiensis* observed in experiment 1 and *ii*) to understand the mechanisms of PCN suppression in inundated conditions in non-amended and amended soil by studying the effect of amendments on hatching, host finding ability and infectivity of J2 and by determining the chemical and biological changes in the soil at more frequent time intervals. Soil collected from the field (same as in experiment 1) was thoroughly mixed with PP, FPP, and L at rates mentioned earlier. These amendments were selected because they caused a significant reduction in the viability of *G. rostochiensis*, in the

first experiment. Inundated non-amended soil was used as a control. Fifteen 2 L-airtight containers were filled with 1.5 L of each of the amendments mixed with soil. This time, 30 cysts of *G. rostochiensis* and 30 cysts of *G. pallida* were placed in separate retrievable nylon mesh bags and buried in the middle of the soil column in the containers. Finally, 400 ml tap water was added to each container. This experiment had 4 treatments and each treatment received 15 replications.

The PCN viability was assessed 14, 17, 21, 24 and 28 days after inundation, using the trehalose-based method as described in experiment 1. This experiment was 4 weeks shorter than experiment 1 because of a significant reduction in viability of *G. rostochiensis* eggs after 4 weeks inundation in combination with amendments. Hatching and infectivity of the eggs and J2 inside the PCN cysts retrieved from the containers were also examined at the 5 time intervals mentioned above. At each observation, cysts were recovered from three containers of each treatment (3×30 cysts). From each bag, 10 cysts were used for the viability assessment and the other 20 cysts were exposed to potato root diffusate (PRD) obtained from cv. Désirée (Turner *et al*., 2009) for a hatching assay; the resulting hatched J2 were used for an assay to determine the host finding ability of the J2 (see section 7.2.4.2). Physical and chemical characteristics of soil were also determined at each time interval (see section 7.2.4.3). This experiment was repeated in time.

7.2.4.1 Hatching assay

The effect of the amendments on the hatching behaviour of J2 of *G. rostochiensis* and *G. pallida* was examined using a hatching assay. PRD was collected from cv. Désirée following the protocol described by Turner *et al*. (2009). At each observation, 20 cysts of each species, *G. rostochiensis* and *G. pallida*, recovered from containers were exposed to PRD (see section 3.4). The samples were maintained at 21°C in a completely randomized design. The number of hatched J2 was counted at weekly intervals over a period of 8 weeks; PRD was renewed at every counting event. After 8 weeks, all cysts were crushed and the number of viable unhatched juveniles inside the cysts was counted visually to enable calculation of the percentage of the total cyst content that hatched during the assay.

7.2.4.2 Host finding ability of treated encysted juveniles

A 23% pluronic gel (F-127, Sigma-Aldrich, Belgium) was prepared following the procedure described by Wang *et al*. (2009). One hundred freshly hatched J2 of *G. rostochiensis* or *G. pallida*, recovered from the hatching assay at week 3 from cysts retrieved from inundated non-amended soil (S+W) and from amended soils (S+W+FPP, S+W+PP, and S+W+L), were mixed thoroughly with the gel before it solidified. Petri dishes (9 cm diam.) were filled with 10 ml of the gel including the 100 J2. A tomato seedling (cv. Moneymaker) was put in the centre of each Petri dish, with the root inside the gel. The Petri dishes were maintained at room temperature for the gel to solidify and then incubated at 22°C in the dark. After 4, 8 and 48h the number of J2 present in the area of 1 cm around the tomato roots was counted. At the last observation, the seedlings were removed from the gel, washed gently and stained with acid fuchsin (Byrd *et al*., 1983) to determine the number of J2 that had penetrated the roots (see section 3.6). This assay had 4 treatments, which were replicated 3 times. The assay was performed only at three observation times (14, 17 and 21 days after inundation) because hatched J2 were not observed at later time points (24 and 28 days).

7.2.4.3 Chemical and biological analysis of soil

For this second experiment, at each of the 5 observation times, soil pH and concentrations of SCFA in the soil water phase extract were determined, as described in experiment 1. The redox potential of the soil was measured at every observation as an indicator of the level of anaerobiosis (Husson, 2013), using platinum (Pt) electrodes and a glass calomel reference electrode (Schott Geräte, Hofheim, Germany). The Pt-electrodes were inserted into inundated amended and non-amended soils up to a depth of 7 cm.

At the end of the experiment, *i.e*., four weeks after inundation, a 100-g soil sample taken from each container was freeze-dried to determine the changes in biomass and composition of the microbial community in anaerobic conditions by analysing phospholipid fatty acid (PLFA) profiles of soil (Denef *et al*., 2007). The extraction of lipids from 6-g freeze-dried samples, the isolation of phospholipids and the methanolysis of the phospholipids were performed as described in chapter 6.

7.3 Data analysis

Experiments 1 and 2 were performed twice. The data obtained from the repeats were combined and used in a single analysis when these data did not differ significantly. Because the results on the viability obtained from the two repeats of experiment 1 were significantly different, these data were not pooled. The percentages of viable eggs measured by each of the viability assessing methods (*i.e.*, visual assessment and the trehalose-based method) in experiment 1 were subjected to a three-way-factorial ANOVA on arcsine-transformed data to determine the effect of the method of assessment, the exposure time and the soil treatments. The non-transformed data were used to produce the graphs.

The data from the viability assessment and the hatching assay obtained in experiment 2 were also arcsine-transformed and then analysed using a three-way-factorial ANOVA to determine the effect of the species, the exposure time, the soil treatment, and their interactions. Data on the host finding ability test (pluronic gel assay) were subjected to the repeated measures ANOVA. The numbers of nematodes inside the roots in the gel assay were compared using one-way ANOVA. The data of the infectivity test, concentration of SCFA, total biomass of PLFA, gases, soil pH and redox potential were also subjected to ANOVA. Differences among the treatments were analysed with Tukey's Honest Significant Difference (HSD) tests and differences were considered significant when $P < 0.05$. Analyses were performed with STATISTICA_10 software (Statsoft, OK, USA).

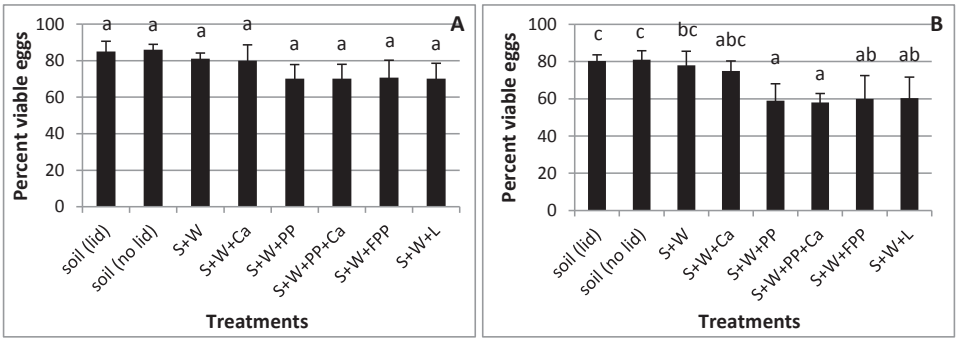
7.4 Results

7.4.1 Experiment 1

7.4.2 Effect of inundation on the viability of *G. rostochiensis*

There was no significant difference between the viability results obtained with visual assessment and the trehalose-based method ($P > 0.05$). Therefore, in both repeated experiments, the effect of the amendments on viability was evaluated using the average data obtained with both methods.

The viability of PCN cyst content was reduced by factor 'treatment' (F = 1544.35; df = 7; $P \leq 0.001$) (Figure 7.2). The reduction increased with time of exposure (F = 945.66; df = 7; $P \leq 0.001$). The interaction 'time' × 'treatment' was significant (F = 51.98; df = 49; $P \leq 0.001$). In the first week, no significant differences were observed between treatments (Figure 7.2 A). After two weeks of exposure of cysts to inundated soil amended with fresh and steamed potato peels or leek, there was a significant reduction in the viability of *G. rostochiensis* (F = 6.92; df = 7; $P \leq 0.001$). The percentage viable eggs in these treatments were reduced to 58% and 60%. Inundation of non-amended soil (S+W) or soil amended with calcium sulphate (S+W+Ca) had no influence on the viability of eggs and J2 after 2 weeks of incubation (Figure 7.2 B). Nevertheless, after 3 weeks, all inundated amended treatments reduced the viability compared with both non-inundated soil controls (in open or closed containers) (Figure 7.2 C) (F = 93.19; df = 7; $P \leq 0.001$). Moreover, all soils amended with steamed and fresh potato peels or leek reduced the viability significantly more than inundation without amendments (S+W) or soil amendment with calcium sulphate only (S+W+Ca). This difference between amendments was also observed at week 4 when the viability of *G. rostochiensis* was reduced to 99.9% in soils amended with fresh and steamed potato peels or leek, while only 50% and 45%, for inundation without amendments (S+W) or soil amended with calcium sulphate (S+W+Ca), respectively (Figure 7.2 D). These treatments caused 72% egg mortality 8 weeks after the start of the experiment (Figure 7.2 H).



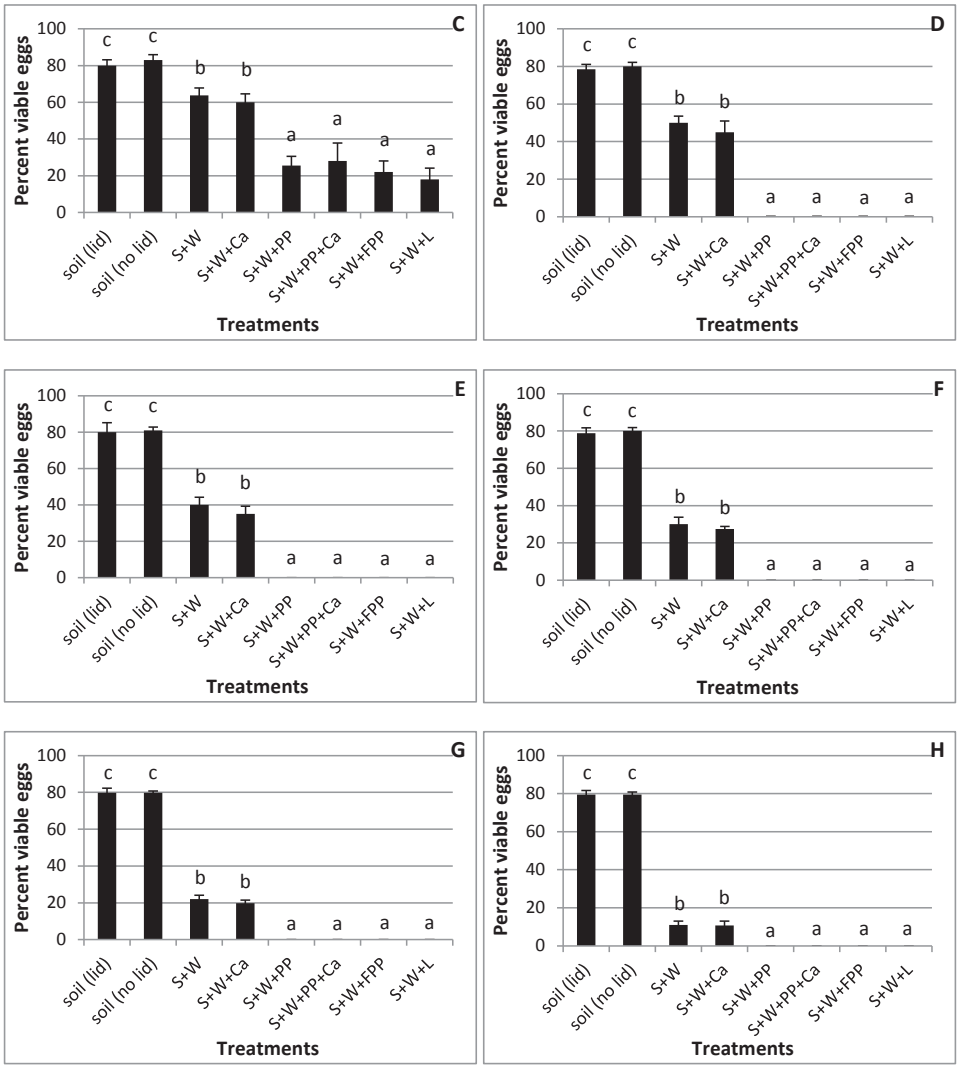


Figure 7.2. Effect of inundation and soil amendments on the viability of *Globodera rostochiensis* eggs inside cysts buried in soil over a period of 1-8 weeks (week 1 (A), week 2 (B), week 3 (C), week 4 (D), week 5 (E), week 6 (F), week 7 (G) and week 8 (H)).Treatments: soil (lid): non-amended non-inundated soil covered with lid; soil (no lid): non-amended non-inundated soil without lid (controls); S+W: inundated non-amended soil; S+W+Ca: inundated soil amended with calcium sulphate; S+W+PP: inundated soil amended with steamed potato peels; S+W+PP+Ca: inundated soil amended with a mixture of steamed potato peels and $CaSO_4$; S+W+FPP: inundated soil amended with fresh potato peels; S+W+L: inundated soil amended leek. Values are the means of 4 replicates. Error bars represent the standard error of the mean. Different letters indicate differences in the percentage of viability between treatments according to Tukey's Honest Significant Difference test ($P < 0.05$).

In the repeat of the experiment, a 43% and 49% reduction in viability of *G. rostochiensis* was already observed in the first week in soil amended with leek (S+W+L) and fresh potato peels (S+W+FPP), respectively, differing significantly from the viability of cysts content in non-inundated soil and inundated soil amended with other materials ($F = 5.33$; $df = 7$; $P \leq 0.001$). In the second week, viability of cysts from soil amended with steamed potato peels and calcium sulphate (S+W+PP+Ca) also showed significant differences with the non-inundated controls ($F = 5.96$; $df = 7$; $P \leq 0.001$). At later observations, viability of *G. rostochiensis* in amended soils with FPP, PP, L and PP+$CaSO_4$ were significantly reduced compared with both controls, non-amended and calcium sulphate amended soil ($P \leq 0.001$) (Data not shown).

7.4.2.1 Effect of inundation on the infectivity of *G. rostochiensis*

The infectivity of PCN was reduced by both 'treatment' ($F = 225.53$; $df = 7$; $P \leq 0.001$) and 'exposure time' ($F = 3.17$; $df = 7$; $P = 0.003$). The effect of the treatment on root penetration of surviving juveniles was independent of the duration of exposure to this treatment ('treatment' × 'exposure time' $F = 0.647$; $df = 49$; $P = 0.96$). After 1 week, fewer J2 (1-63) were observed in potato roots exposed to cysts of *G. rostochiensis* recovered from inundated soil - amended or non-amended - compared with non-inundated soils (199-288) ($F = 5.96$; $df = 7$; $P \leq 0.001$). However, the maximum decrease in infectivity was found when FPP, PP, PP+$CaSO_4$, and L were added to the soil (S+W+PP, S+W+FPP, S+W+PP+Ca, and S+W+L) (Figure 7.3).

After three weeks, no juvenile was observed in potato roots exposed to cysts recovered from inundated soil amended with the different plant wastes (Figure 7.3). Nevertheless, in the roots exposed to cysts recovered from inundated non-amended soil (S+W) and soil where only calcium sulphate was added (S+W+Ca), a few juveniles (3-5 juveniles of different stages, respectively) were observed up to 4 weeks after exposure. However, 150-185 juveniles retrieved from controls were detected inside the roots until the end of the experiment (Figure 7.3).

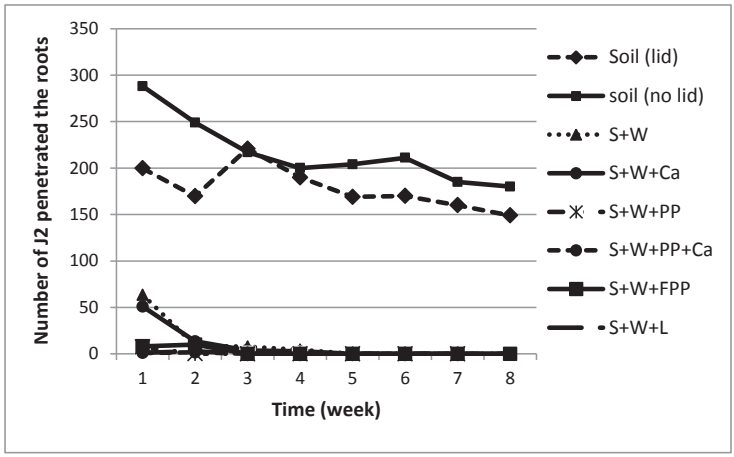


Figure 7.3. Effect of eight treatments on the infectivity of encysted second-stage juveniles of *Globodera rostochiensis* recovered weekly from containers over a period of 8 weeks on potato roots grown in closed plastic containers. Treatments: soil (lid): non-amended non-inundated soil covered with lid; soil (no lid): non-amended non-inundated soil without lid (controls); S+W: inundated non-amended soil; S+W+Ca: inundated soil amended with calcium sulphate; S+W+PP: inundated soil amended with steamed potato peels; S+W+PP+Ca: inundated soil amended with a mixture of steamed potato peels and $CaSO_4$; S+W+FPP: inundated soil amended with fresh potato peels; S+W+L: inundated soil amended leek.

Extraction of J2 from sand in the closed containers at the end of each observation point showed that exposure of *G. rostochiensis* cysts to inundated soil inhibited the hatching. At all observations, fewer hatched J2 were retrieved from inundated soils (S+W) than from controls ($P \leq 0.001$). No J2 were detected in the sand infested with cysts recovered from inundated soils amended with FPP, PP, PP+$CaSO_4$, and L, after two weeks. However, J2 were observed in sand infested with cysts treated by inundation alone (S+W) (19 J2) or with addition of $CaSO_4$ (S+W+Ca) (15 J2) up to 4 weeks. Hatched J2 were observed in the sand infested with cysts recovered from controls till the end of the experiment (8 weeks after inundation).

The repeat of this experiment also confirmed that the number of *G. rostochiensis* J2 penetrating potato roots, recovered from cysts exposed to amendments, was reduced by all amendments at all time-points ($P \leq 0.001$).

7.4.2.2 Gas measurements

All gases were expressed as a percentage of total volume of gases produced in the airtight containers, except for H_2S, which was measured in ppm. One week after incubation, a significant

decrease in the O_2-concentration ($F = 28.4$; $df = 7$; $P \leq 0.001$) and an increase in CO_2-level was registered ($F = 59.51$; $df = 7$; $P \leq 0.001$) in S+W+PP, S+W+FPP, S+W+PP+Ca and S+W+L compared with non-inundated controls and inundated soil with no amendment (S+W) and calcium sulphate amended soil (S+W+Ca).

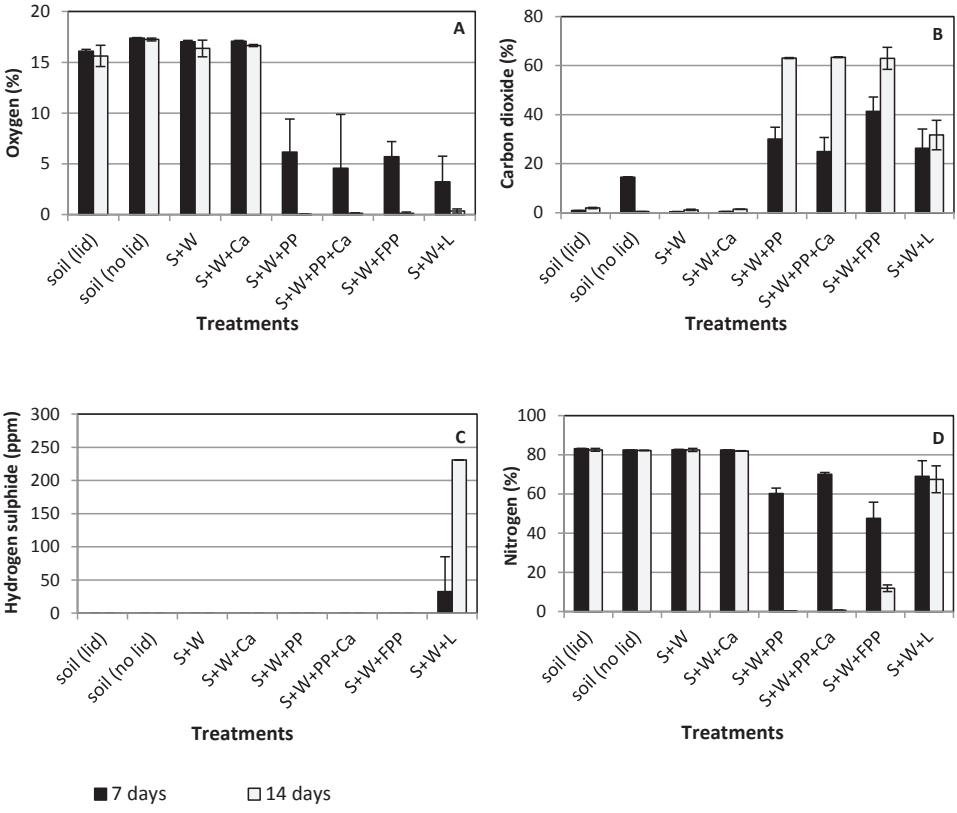


Figure 7.4. Effect of soil treatment on the concentration of oxygen (A), carbon dioxide (B), hydrogen sulphide (C) and nitrogen (D) in containers filled with soil (lid): non-amended non-inundated soil covered with lid; soil (no lid): non-amended non-inundated soil without lid (controls); S+W: inundated non-amended soil; S+W+Ca: inundated soil amended with calcium sulphate; S+W+PP: inundated soil amended with steamed potato peels; S+W+PP+Ca: inundated soil amended with a mixture of steamed potato peels and $CaSO_4$; S+W+FPP: inundated soil amended with fresh potato peels; S+W+L: inundated soil amended leek 7 and 14 days after the start of experiment 1. Error bars represent the standard deviation of the mean.

Two weeks after the start of the experiment, the concentration of O_2 decreased from 15-17% in non-inundated controls to 0.02%, 0.1%, 0.1% and 0.4% in inundated amended soils with

PP, FPP, PP+Ca and L, respectively (Figure 7.4 A). Inversely, there was an increased CO_2-concentration from 0.5-1.9% in non-inundated controls to 63%, 63%, 63% and 32% in inundated amended soils with PP, FPP, PP+Ca and L, respectively (Figure 7.4 B). 231 ppm hydrogen sulphide (H_2S) was only detected in containers filled with leek (S+W+L) (Figure 7.4 C). Methane (CH_4) and ammonia (NH_3) were not detected in any treatment. There was a significant amount of nitrogen (N_2) in all containers one week after incubation. However, two weeks after the initiation of the experiment, the N_2 concentration drastically dropped from 82% in non-inundated controls to 0.3%, 0.7%, 12% and 67% in soils amended with PP, PP+Ca, FPP and L, respectively (Figure 7.4 D).

7.4.2.3 Short chain fatty acids and pH

Higher concentrations of SCFA, including acetic, butyric, propionic and iso-butyric acids were detected in soils amended with PP (184.4 µmol ml^{-1}), FPP (180.8 µmol ml^{-1}) and PP+Ca (124.4 µmol ml^{-1}) ($F = 59.06$; $df = 7$; $P \leq 0.001$) than in other treatments (Table 7.1). Leek amended soil contained less SCFA (27.4 µmol ml^{-1}) than these amended soils. 14.6 µmol ml^{-1} SCFA was also detected in non-amended inundated soil (S+W), which was significantly lower than that in amended soils mentioned above. No SCFA were detected in non-inundated soils (soil (lid) and soil (no lid)) or in inundated soil amended with calcium phosphate (S+W+Ca) (Table 7.1). In soils amended with PP, FPP, PP+Ca and L, the highest concentrations were found for acetic and, butyric acid and to a lesser extent for propionic acid (Table 7.1). There was a negligible amount of valeric and isovaleric acid.

Concentrations of SCFA were negatively correlated with soil pH (Pearson coefficient of -0.82, -0.85, -0.90 and -0.80 for S+W+FPP, S+W+PP, S+W+PP+Ca and S+W+L, respectively). Soil pH was reduced from 7 in controls, to 2.8, 3.0, 3.0 and 3.10 in soils amended with crop wastes, FPP, L, PP, and PP+$CaSO_4$, respectively (Table 7.1). There was no significant change in the soil pH in inundated soil with no amendment (pH 7.25) or amended with calcium sulphate (pH 7).

Table 7.1. Soil pH and concentrations of short chain fatty acids produced over a period of 8 weeks in inundated soils amended with different types of products consisting of potato peels, leek or calcium sulphate, or left non-inundated. Data are the means ± standard deviation of four replicates (n = 4).

Treatment*	pH	Acetic acid (µmol ml^{-1})	Propionic acid (µmol ml^{-1})	Butyric acid (µmol ml^{-1})	Isobutyric acid (µmol ml^{-1})	Valeric acid (µmol ml^{-1})	Isovaleric acid (µmol ml^{-1})	Total SCFA
Soil (no lid)	7.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 c
Soil (lid)	7.04 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 c
S+W	7.25 a	10.70 ± 10.06 a	1.20 ± 1.12 a	2.64 ± 2.27 a	0.04 ± 1.27 a	0.00 a	0.00 a	14.58 ± 13.31 bc
S+W+Ca	7.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 c
S+W+PP	3.05 b	75.37 ± 12.91 c	20.06 ± 4.53 c	84.23 ± 14.23 c	4.29 ±0.42 b	0.18 ± 0.05 a	0.23 ± 0.06 a	184.35 ± 31.90 a
S+W+PP+Ca	3.10 b	45.30 ± 6.90 bc	14.08 ± 3.39 bc	60.96 ± 16.77 b	3.62 ± 1.43 b	0.22 ± 0.05 a	0.21 ± 0.06 a	124.39 ± 28.05 a
S+W+FPP	2.80 b	86.21 ± 18.15 c	9.44 ± 2.40 b	81.76 ± 17.85 c	2.20 ± 0.10 b	0.38 ± 0.10 a	0.80 ± 0.09 a	180.79 ± 38.24 a
S+W+L	3.01 b	20.87 ± 14.03 b	1.88 ± 0.67 a	4.43 ± 5.39 a	0.08 ± 0.08 a	0.00 a	0.17 ± 017 a	27.43 ± 17.46 b

*soil (lid) = non-amended non-inundated soil covered with lid; soil (no lid) = non-amended non-inundated soil without lid (controls); S+W = inundated non-amended soil; S+W+Ca = inundated soil amended with calcium sulphate; S+W+PP = inundated soil amended with steamed potato peels; S+W+PP+Ca = inundated soil amended with a mixture of steamed potato peels and $CaSO_4$; S+W+FPP = inundated soil amended with fresh potato peels; S+W+L = inundated soil amended leek.
Different letters in each column indicate significant differences ($P < 0.05$) among the treatments.

7.4.3 Experiment 2: Mechanisms of *G. rostochiensis* and *G. pallida* suppression in inundated amended soils

7.4.3.1 Effect of inundation on the viability of *G. rostochiensis* and *G. pallida*

The viability of eggs and J2 of *G. rostochiensis* and *G. pallida* was significantly lower in amended inundated soils (S+W+L, S+W+PP and S+W+FPP) than in non-amended inundated soil (S+W) at all observations ($P \leq 0.001$) (Figure 7.5). However, no significant differences were observed among the three amended treatments at all observations. The viability decreased with the exposure time ($F = 631.62$; $df = 4$; $P \leq 0.001$), but the effect of exposure time on viability varied with treatment (interaction 'treatment' × 'exposure time', $F = 13.49$; $df = 12$; $P \leq 0.001$).

No differences were found for the main factor 'species' ($F = 0.006$; $df = 1$; $P = 0.94$) or for interactions 'treatment' × 'species' ($F = 0.04$; $df = 3$; $P = 0.99$), 'species' × 'exposure time' ($F = 0.146$; $df = 4$; $P = 0.96$) and 'treatment' × 'species'× 'exposure time' ($F = 0.29$; $df = 12$; $P = 0.99$).

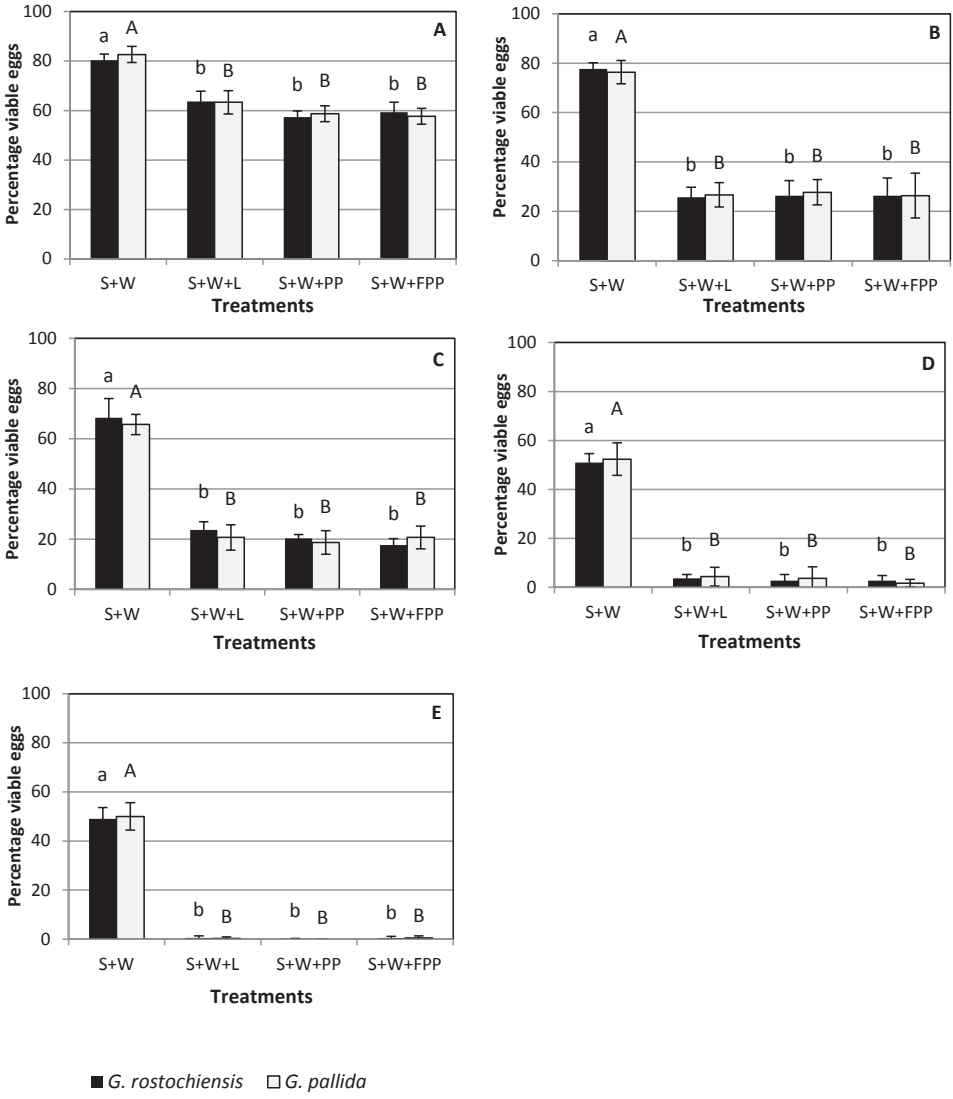


Figure 7.5. Effect of four treatments on viability of encysted eggs of *Globodera rostochiensis* and *G. pallida* kept in inundated soil for different times 14 (A), 17 (B), 21 (C), 24 (D) and 28 (E) days after inundation. Treatments: S+W: inundated non-amended soil; S+W+L: inundated soil amended with leek; S+W+PP: steamed potato peels and S+W+FPP: fresh potato peels. Values are the means of combined data of two experiments repeated in time (n=6). Error bars represent the standard error of the mean. Within a nematode species, bars headed by a different letter, lowercase (*G. rostochiensis*) or uppercase (*G. pallida*), are significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).

Fourteen days after the start of the experiment, the viability of encysted eggs and juveniles of *G. rostochiensis* in amended soils was reduced to 25-28%, while in non-amended soil (S+W) viability was only reduced to 77% ($F = 28.29$; $df = 3$; $P \leq 0.001$) (Figure 7.5 A). The exposure time influenced the viability of PCN ($F = 631.62$; $df = 4$; $P \leq 0.001$). The number of viable eggs of *G. rostochiensis* was reduced in the succeeding observations. After 28 days of inundation, the viability of eggs and juveniles of this species was reduced by more than 99% in inundated soils with amendments. Yet, only 49% mortality was observed in non-amended inundated soil (Figure 7.5 E).

Similar trends of reduction in viability were observed for *G. pallida* in inundated conditions (Figure 7.5). Fourteen days after the start of the experiment significant differences were observed between the amended treatments and non-amended soil (Figure 7.5 A) ($F = 30.56$; $df = 3$; $P \leq 0.001$). Observations at next samplings also showed significant reduction in viability of encysted eggs of this species in amended inundated soil compared with that in non-amended inundated soil ($P \leq 0.001$). At the end of the experiment, S+W+PP, S+W+L and S+W+FPP caused 100%, 99.7% and 99.5% mortality in eggs and juveniles of *G. pallida*. However, only 50% egg mortality was observed in non-amended treatment (S+W) 28 days after inundation (Figure 7.5 E).

7.4.3.2 Effect of inundation and amendments on the hatching of *G. rostochiensis* and *G. pallida*

At each observation time, the total percentage hatch of *G. rostochiensis* and *G. pallida* was reduced with increasing exposure time ($F = 498.07$; $df = 4$; $P \leq 0.001$). It was also affected by the factor 'treatment' ($F = 286.46$; $df = 3$; $P = 0.004$). However, hatching was not influenced by the factor 'species' ($F = 0.42$; $df = 1$; $P = 0.52$) or by the interaction 'treatments' × 'species' ($F = 0.12$; $df = 3$; $P = 0.95$), the interaction 'species' × 'time' ($F = 0.33$; $df = 4$; $P = 0.86$), and by the interaction 'treatment' × 'species' × 'exposure time' ($F = 0.29$; $df = 12$; $P = 0.99$).

A comparison of the total percentage hatch (over an 8-week hatching assay) of *G. rostochiensis* J2 retrieved from containers at different time intervals (14, 17, 21, 24 and 28 days after inundation) showed significant differences between the three amended treatments and the non-amended control ($P \leq 0.001$). Fewer J2 of *G. rostochiensis* hatched from cysts recovered

from inundated amended soils 14 days after inundation than from non-amended soil (S+W) (F = 15.60; df = 3; P = 0.001); between 38% and 41% of the cyst content hatched when fresh and steamed potato peels or leek were added, while 70% of the J2 hatched when no amendments had been added (Figure 7.6 A).

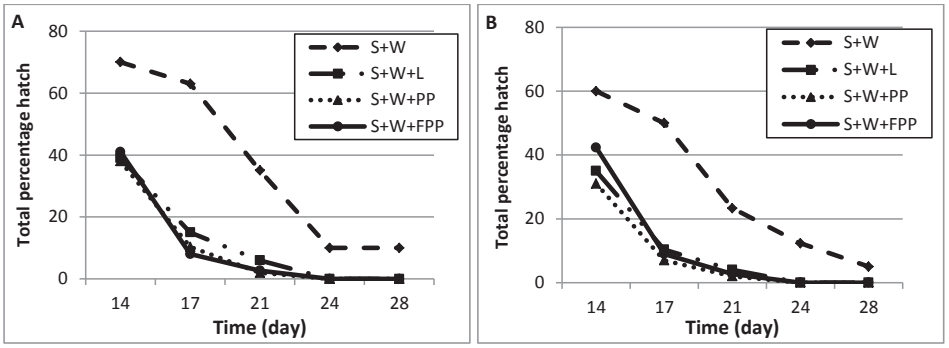


Figure 7.6. Influence of soil treatment and exposure time on hatching of juveniles from cysts of *Globodera rostochiensis* (A) and *G. pallida* (B) at different time intervals (14, 17, 21, 24 and 28 days) after the start of the experiment. Soils were inundated non-amended: S+W or amended with leek: S+W+L, steamed potato peels: S+W+PP and fresh potato peels: S+W+FPP.

Hatching decreased with time for all treatments. No J2 hatched from cysts that were incubated for 24 and 28 days in amended inundated soils, while still 10% of the cyst content hatched from cysts retrieved from non-amended soil (S+W) 24 and 28 days after inundation (Figure 7.6 A).

General trends of the data on the hatch percentage were similar for *G. pallida*. A significant reduction in the hatching was observed for the juveniles originating from cysts incubated for 14 days in amended inundated conditions compared with non-amended soil (F = 17.68; df = 3; P = 0.001). Hatched J2 were not observed from cysts recovered from the amended soil 24 days after the start of the experiment (Figure 7.6 B). However, 28 days after inundation, still 5% of J2 hatched from cysts retrieved from non-amended inundated soil.

7.4.3.3 Effect of inundation on the host finding ability of *G. rostochiensis* and *G. pallida*

The host finding assay was performed only at three time points after the start of the inundation test (14, 17 and 21 days after inundation) because hatched J2 were not observed at

later time points (24 and 28 days). The host finding ability of *G. rostochiensis* J2 recovered from cysts, which were incubated for 14, 17 and 21 days in inundated conditions, was not influenced by the amendments ($F = 9.79$; $df = 3$; $P = 0.10$).

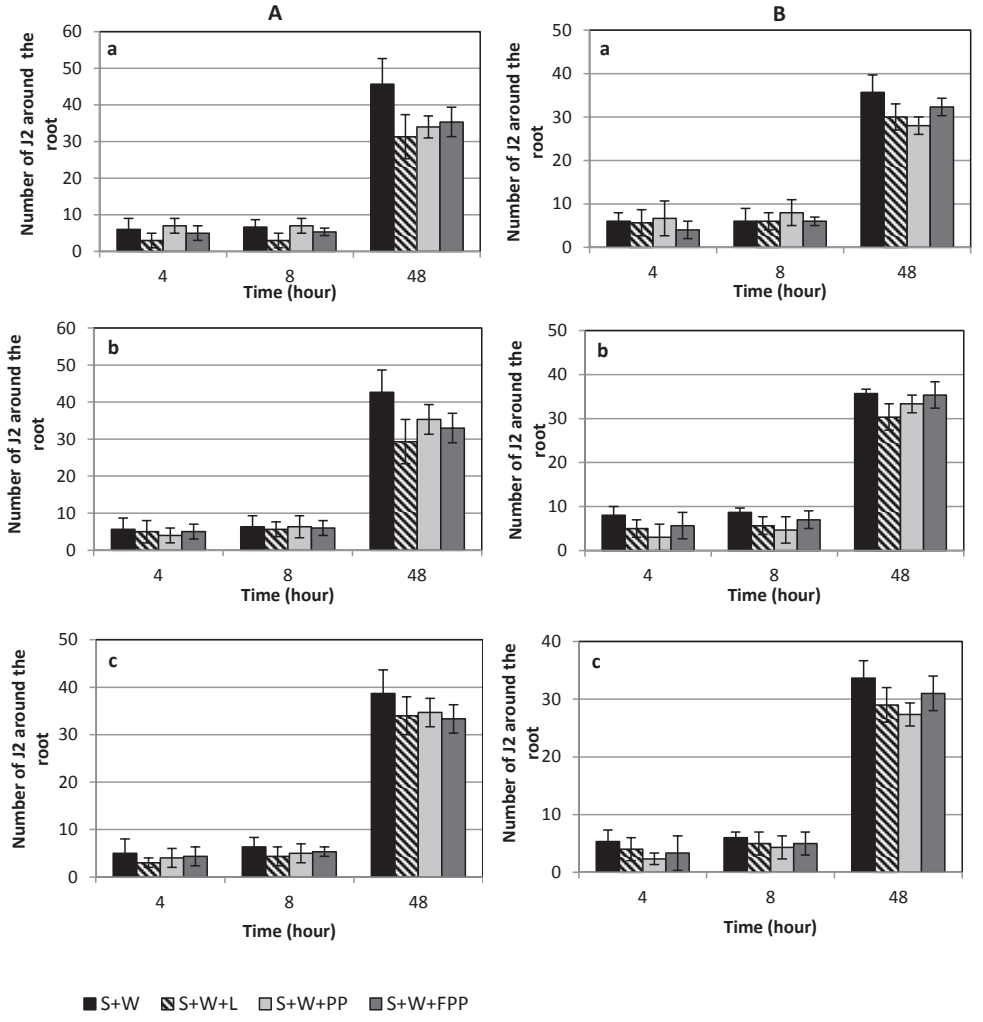


Figure 7.7. Movement of second-stage juveniles of *Globodera rostochiensis* (A) and *G. pallida* (B) obtained from treated cysts recovered from containers filled with S+W: inundated non-amended soil; S+W+L: inundated soil amended with leek; S+W+PP: steamed potato peels and S+W+FPP: fresh potato peels towards tomato roots in pluronic gel 14 days (a), 17 days (b) and 21 days (c) after soil treatment.

Forty eight hours after the start of the pluronic gel assay, the number of *G. rostochiensis* J2 around the tomato roots varied between 29 for J2 recovered from cysts remained in inundated amended soils and 45 for those from cysts recovered from non-amended inundated soil (control), irrespective of the incubation condition (amended or non-amended) and of the duration of inundation (Figure 7.7 A). The duration of inundation did not influence the movement of the J2 towards roots ($F = 1.81$; $df = 2$; $P = 0.17$). The number of *G. rostochiensis* J2 in the vicinity of tomato roots increased with time of observation (4, 8 and 48h) ($F = 475.70$; $df = 2$; $P \leq 0.001$) (Figure 7.7 A).

Host finding ability was not influenced by 'species' ($F = 5.27$; $df = 1$; $P = 0.06$); similar observations were made for *G. pallida*. With increasing time of observation (from 4h to 48h), more J2 were counted close to the root tips ($F = 259.38$; $df = 2$; $P \leq 0.001$). Amendments had no significant impact on the number of J2 attracted to the host roots ($F = 1.73$; $df = 3$; $P = 0.17$). The number of J2 in the vicinity of the root tips ranged between 28 and 34 for the J2 recovered from cysts incubated in amended soils and those recovered from non-amended soil (control) (Figure 7.7 B). No effect of the duration of inundation (14, 17 and 21 days) was observed on the movement of the J2 of *G. pallida* towards tomato roots ($F = 1.78$; $df = 2$; $P = 0.18$).

Penetration of tomato roots by J2 of *G. rostochiensis* 14, 17 or 21 days after inundation did not differ between amended and non-amended soils ($F = 1.87$; $df = 3$; $P = 0.21$, $F = 0.89$; $df = 3$; $P = 0.49$ and $F = 8.28$; $df = 3$; $P = 0.08$, respectively) (Figure 7.8 A). Amending soil with leek (S+W+L), steamed (S+W+PP) or fresh potato peels (S+W+FPP) for 14, 17 and 21 days did not influence the ability of *G. pallida* J2 to penetrate the roots ($F = 1.24$; $df = 3$; $P = 0.36$, $F = 2.96$; $df = 3$; $P = 0.06$ and $F = 6.47$; $df = 2$; $P = 0.10$, respectively) (Figure 7.8 B).

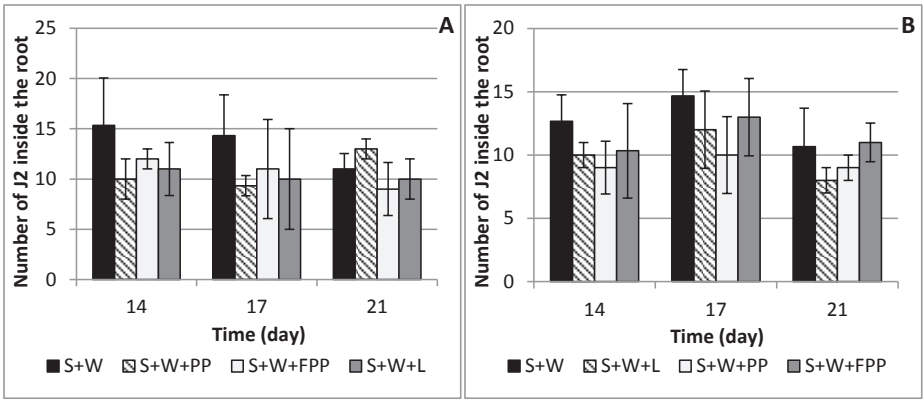


Figure 7.8. Root penetration of second-stage juveniles of *Globodera rostochiensis* (A) and *G. pallida* (B) in tomato roots obtained from treated cysts recovered from containers filled with S+W: inundated non-amended soil; S+W+L: inundated soil amended with leek; S+W+PP: steamed potato peels and S+W+FPP: fresh potato peels in tomato roots in pluronic gel 14, 17 and 21 days after inundation. Values are the mean of three replicates (n=3). Error bars represent the standard deviation of the mean.

7.4.3.4 Short chain fatty acids (SCFA)

Fourteen days after the start of the experiment, SCFA were already detected in the water phase in the containers filled with amendments (S+W+FPP, S+W+PP and S+W+L). However, SCFA were only detected in the non-amended soil (S+W) 24 days after the experiment initiated (Figure 7.9). At all observations, amendments increased the production of volatile fatty acids in inundated conditions compared with the non-amended soil ($P \leq 0.001$). Highest concentrations of total SCFA were found in the soil amended with steamed potato peels 28 days after inundation. Acetic acid, butyric acid and to a lesser extent propionic acid were the most abundant SCFA present in all amended soils (data not shown).

7.4.3.5 Redox potential

At a pH of 7, redox potentials >414 mV indicate oxic (*i.e.*, aerobic) soil conditions, redox potentials 414–120 mV indicate suboxic soil conditions and redox potentials <120 mV represent anoxic (anaerobic) soil conditions (Essington, 2004). Soil redox potentials decreased with increased the duration of inundation. At all observations, amendments reduced the redox potential ($P < 0.001$) (Figure 7.10).

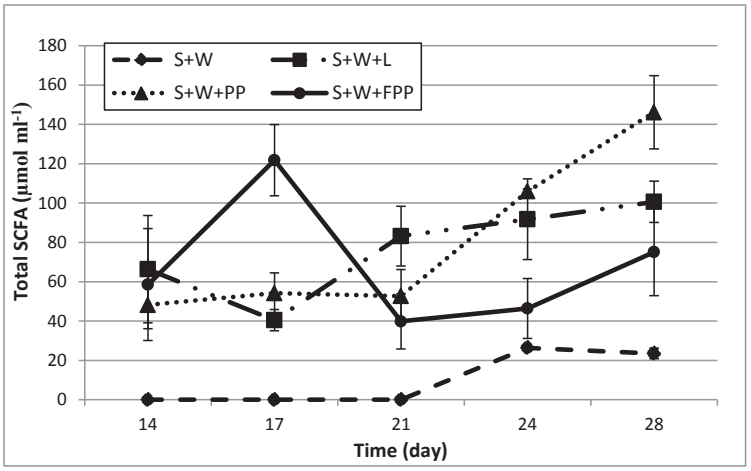


Figure 7.9. Concentrations of all short chain fatty acids (SCFA) measured in the soil water phase extract in containers filled with inundated soils, either non-amended (S+W) or amended leek (S+W+L); steamed potato peels (S+W+PP) or fresh potato peels (S+W+FPP), from 14 up to 28 days after soil treatment.

The redox potential of soil was 485 ± 3.21 mV prior to soil inundation, indicating the oxic (aerobic) condition in the soil. However, a significant reduction in the redox potential was observed already one day after inundation of soil amended with steamed potato peels (172.11 mV) and leek (110.53 mV) compared with non-amended soil (343.84 mV) (Figure 7.10). After 28 days, the lowest redox potential was observed for the soil amended with fresh potato peels (-382.76 mV).

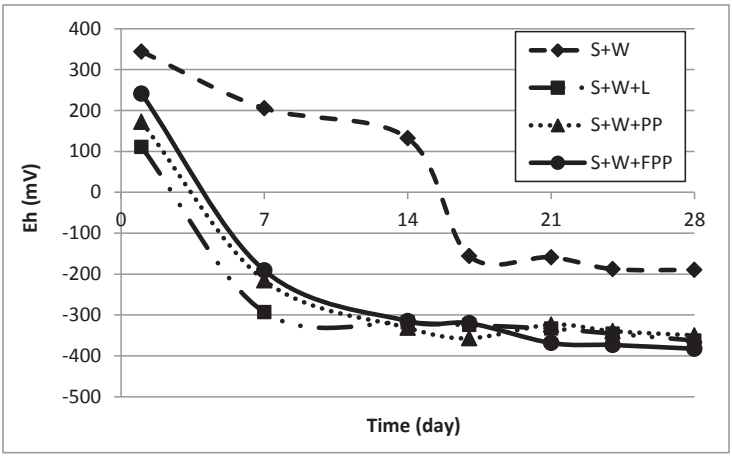


Figure 7.10. Redox potential measured in containers filled with S+W: inundated non-amended soil; S+W+L: inundated soil amended with leek; S+W+PP: steamed potato peels and S+W+FPP: fresh potato peels, 1, 7, 14, 17, 21, 24 and 28 days after inundation. Values are the means of three replicates (n = 3).

7.4.3.6 Phospholipid fatty acid (PLFA) composition of the soil microbiota

The total concentration of PLFAs differed among treatments (F = 32.39; df = 3; P < 0.001). More PLFAs were detected in soils amended with potato peels (S+W+PP and S+W+FPP) than in soils amended with leek or non-amended soils (Table 7.2).

For the marker PLFAs of selected microbial groups (bacteria, fungi, and actinomycetes), significant differences were observed among treatments (P < 0.001). However, the total biomass of arbuscular mycorrhizal fungi did not differ among treatments. The highest bacterial and fungal PLFAs were found in soil amended with steamed potato peels (S+W+PP) (Table 7.2). No significant differences were observed for selected microbial groups in leek amended soil, except for gram negative bacteria.

Table 7.2. Mean absolute abundance (nmol g^{-1}) ± standard deviation of organisms detected in amended and non-amended soil 28 days after soil inundation.

Organism	S+W	S+W+PP	S+W+FPP	S+W+L
Non-specific bacteria	10.53 ± 0.65 a	31.40 ± 2.44 b	22.67 ± 4.59 b	12.98 ± 1.23 a
Gram positive bacteria	8.78 ± 0.49 a	24.66 ± 1.64 c	14.27 ± 2.40 bc	7.49 ± 0.51 a
Actinomycetes	3.39 ± 0.10 a	1.58 ± 0.19 b	1.33 ± 0.17 b	2.22 ± 0.14 a
Gram negative bacteria	8.90 ± 0.45 a	23.17 ± 11.41 c	17.049 ± 0.36 b	14.37± 2.35 bc
AMF	1.34 ± 0.11 a	0.53 ± 0.10 a	0.40 ± 0.05 a	0.84 ± 0.17 a
Fungi	1.92 ± 0.10 a	4.44 ± 0.69 b	1.71 ± 0.39 a	2.40 ± 0.17 ab
Total biomass	34.86 ± 1.60 a	85.78 ± 12.71 c	57.42 ± 3.49 b	40.30 ± 4.31 a

S+W = inundated non-amended soil; S+W+PP = inundated soil amended with steamed potato peels; S+W+FPP = inundated soil amended with fresh potato peels; S+W+L = inundated soil amended with leek; AMF = arbuscular mycorrhizal fungi. Different letters in each row indicate significant differences ($P < 0.05$) among the treatments.

7.5 Discussion

This study investigated the influence of agro-industrial waste products on the efficacy of inundation to reduce the survival of the potato cyst nematodes, *G. rostochiensis* and *G. pallida* in waste soils. The mechanisms involved in the suppression of both PCN species in inundated conditions of non-amended or amended soil were also examined.

Both PCN species lost their viability over prolonged periods (4 weeks) of inundation combined with amendments. Inundating soil amended with agricultural waste, *viz*. steamed and fresh potato peels and the green leaves of leek, resulted in a significant loss of viability of both PCN species (more than 99.5%) within a period of 28 days. Inundation of PCN contaminated soil without incorporation of organic matter led to 72% decrease over a period of 8 weeks. Obviously, these organic amendments accelerated the disinfestation process of the PCN contaminated soil. These findings corroborate those of van Overbeek *et al*. (2014) who reported a significant decline (99.4% after 12 weeks inundation) in viability of *G. pallida* in inundated soil amended with grass or Herbie 7025. Spaull *et al*. (1992) observed a marked reduction in viability of *G. pallida* (97.7%) in soil amended with a carbon source (starch) and calcium sulphate after 13 days inundation compared with that in inundated non-amended soil (16%). They reported 99.7% egg mortality in soil without amendments only 14 weeks after inundation. However, unlike the other amendments tested, calcium sulphate alone did not enhance the mortality of PCN. Neither did it improve the effects of steamed potato peels.

Both methods of assessing viability of eggs and juveniles of *G. rostochiensis* yielded similar results; there was no significant difference between the visual observation and the trehalose-based method. However, the trehalose-based method is a fast, cheap and objective method compared with the visual assessment. The trehalose-based method was able to assess the PCN viability even within a short period (1 week) after the death of the cysts content, further confirming the validity of this method. In previous studies, this method was evaluated by determining the viability of old dead cysts collected from fields and cysts artificially killed by heat (van den Elsen *et al*., 2012).

When trying to unravel the mechanisms of the PCN suppression, it was observed that inundation alone reduced the redox potential of the soil markedly. Adding amendments (steamed and fresh potato peels or leek) prior to inundation further decreased soil redox potential (up to -382 mV) and pH (*ca* 3). The concentration of O_2 dropped dramatically from 15-17% in non-inundated soils to 0.02-0.4% in amended soils whilst CO_2 levels increased from 0.5-1.9% in non-inundated soils to 63% in amended soils. This suggests that incorporation of amendments increased the growth of aerobic microorganisms, which consumed O_2 and generated the organic acids (Momma, 2008). Hence, a noticeable increase in concentrations of SCFA was observed in the amended soil, particularly in inundated soils amended with potato peels. In experiment 2, the viability of PCN significantly declined with decreasing the redox potential and increasing amounts of SCFA. According to McElderry *et al*. (2005), a lower pH has a strong positive influence on the nematicidal activity of SCFA due to the increase of the percentage of non-ionized forms of these organic acids, which are more toxic to nematodes as they can penetrate the cell membrane and kill nematodes. Therefore, the faster decline of PCN viability in amended inundated soil could be attributed to non-ionized forms of acetic, butyric and propionic acids produced at the very low soil pH-values that were measured. Decreasing PCN viability coinciding with increasing SCFA and decreasing soil pH, corroborates previous observations by Browning *et al*. (2004), McElderry *et al*. (2005) and Runia *et al*. (2014). Apart from having an effect on the production of non-ionized SCFA, the decrease in soil pH on its own probably contributed to nematode death. McElderry *et al*. (2005) reported 80% mortality of *Tylenchorhynchus* spp. incubated for 7 days in soil acidified with HCl with a pH of 3.5.

Short chain fatty acids are known to have nematicidal effects (Taba *et al*., 2006; Mahran *et al*., 2008). Organic acids produced during the decomposition of organic matter, such as rice bran (Taba *et al*., 2006), rye (*Secale cereale*), timothy grass (*Phleum pratense*), glucose and unsulfured molasses (Patrick *et al*., 1965; Sayre *et al*., 1965), are toxic to plant-parasitic nematodes. The suppression of several nematode species (*Tylenchorhynchus* spp., *Hoplolaimus* spp., *Meloidogyne incognita*) in flooded soil amended with cornmeal was attributed to the production of butyric and propionic acids (Hollis & Rodríguez-Kábana, 1966). The mortality of *G. pallida* in soil amended with grass and Herbie was partially associated with the production of organic acids in anaerobic conditions (Runia *et al*., 2012). In a pot study examining anaerobic soil disinfestation using wheat bran, the production of both acetic and butyric acid was reported (Momma *et al*., 2006). Incorporation of residues of cruciferous plants into soil in pots and incubated at 38°C (day) and 27°C (night) reduced the survival of *M. incognita*, *Sclerotium rolfsii* and *Pythium ultimum*; the production of toxic volatile compounds was suggested to be responsible for the decline of the pathogens population (Stapleton & Duncan, 1998).

The rate of SCFA required to attain nematode mortality varies among the trophic groups of nematodes. Plant-parasitic nematodes are the most sensitive, followed by fungivorous, entomogenous, and bacterivorous nematodes (Browning *et al*., 2004). McElderry *et al*. (2005) reported that butyric and propionic acid were both effective against *Tylenchorhynchus* spp. at a very low concentration of 8.8 mg kg^{-1} soil. Butyric acid at high concentrations (0.88-8.8 g kg^{-1} soil) caused a significant reduction in survival of *Pratylenchus penetrans*, *M. hapla* and *M. incognita* (Browning *et al*., 2004).

To better understand the mechanisms of nematode suppression in inundated soils, changes in the microbial community in non-amended and amended inundated soils were identified using PLFA analysis. The results showed an increase in the bacterial biomass of the inundated soil amended with fresh and steamed potato peels, compared with non-amended soil. Therefore, the reduction in viability of PCN in soil amended with the potato peels can be partially associated with the enhancement of microbial biomass and activity. An increase in populations of microorganisms in inundated soils amended with fresh and steamed potato peels, as well as the higher amounts of organic acids, indicate that the microbial soil community requires organic matter to produce the nematicidal SCFA. Most studies showed that flooding can

strongly impact on soil microbial communities (Bossio & Scow, 1995). It has also been shown that populations of soil microorganisms such as fungi, bacteria and actinomycetes, generally decline in inundated conditions (Mitchell & Alexander, 1962; Unger *et al*., 2009). However, the microbial activity of the anaerobic bacteria belonging to clostridial group, *e.g*. *Clostridium* spp. increases in such conditions (Johnston, 1959; Mowlick *et al*., 2013). SCFA like acetic acid or butyric acid produced by these bacteria were supposed to contribute to pathogen inactivation (Momma *et al*., 2006). In this study, amendments reduced the population of actinomycetes, whilst an increase in the populations of bacteria was observed. In leek-amended soil, a significant increase was observed only in the total biomass of gram-negative bacteria. This might suggest the presence of sulphate-reducing bacteria, which produce the toxic H_2S in the soil. The microorganisms present in inundated soils were not identified at higher taxonomic levels, because the PLFA analysis does not allow the identification of individual species of bacteria or fungi. In-depth genomics-based analyses are needed to shed more light on the potential contribution of individual species in the suppressive capacity of inundated amended soils.

The nematode suppression in leek-amended treatments may also be attributed to the generation of other toxic compounds: those containing sulphur. Arnault *et al*. (2004) showed that different sulphur-containing compounds (disulphides) are the degradation products of *Allium* plants, and are toxic to nematodes. Dipropyl disulphide (DPDS), diallyl disulphide (DADS) and dimethyl disulphide (DMDS) are the main degradation products of leek, onion and garlic. Their toxicity for nematodes and insects is known to be equal to that of methyl bromide. Unfortunately, in this study, the presence of these compounds was not investigated. The incorporation of leek into the soil might have increased the sulphate-reducing bacteria in the soil; hence, generating nematicidal hydrogen sulphide (H_2S). Yet, the presence of toxic H_2S in leek-amended soil can partly explain the nematode suppression in this treatment. However, despite the detection of H_2S in the leek-amended soil in experiment 1, this gas was not found in experiment 2. Significant amounts of SCFA were not always detected in the leek amended soil. This might be due to the variability in the different batches of leek used in different experiments and the time of gas measurements. Nevertheless, the PCN viability was still reduced in the leek amended soil without the generation of high amounts of H_2S and SCFA. Therefore, the reduction in soil redox potential and pH can be the major factors causing the egg mortality. Spaull *et al*. (1992) evidenced that the production of H_2S in anaerobic condition can enhance *G. pallida*

decline in flooded soil. It was also shown that the addition of molasses increased the populations of sulphate-reducing bacteria resulting in production of toxic H_2S (Rodríguez-Kábana *et al*., 1965; Teclu *et al*., 2009).

There was a significant decline of PCN viability in non-amended but inundated soil over a longer time course (8 weeks instead of 4 weeks in amended inundated soils) - despite the negligible amounts of SCFA in non-amended soil. These small amounts of SCFA were probably due to organic contamination of the soil. This decline in viability without amendments demonstrates the effect of other factors in nematode suppression besides SCFA, *e.g*., the depletion of O_2 in an anaerobic soil. Fourteen days after inundation, the redox potential in the non-amended soil was 132 mV, indicating the suboxic soil condition (Essington, 2004). The anoxic (anaerobic) condition was reached 17 days after inundation. As the O_2-concentration decreased in the soil, so did the nematode viability. Earlier studies on the influence of soil oxygen on the survival of plant-parasitic nematodes repeatedly showed a decrease in nematode survival as soon as soil oxygen dropped (Stolzy *et al*., 1960; Van Gundy & Stolzy 1963; Spaull *et al*., 1992).

To understand the modes of action of the amendments on PCN, hatching, host finding ability and root penetration of the J2 was explored. The hatching assay revealed a significant reduction of the emergence of J2 from cysts previously exposed to anaerobic soil conditions. Comparing the results obtained from the viability assessments and the hatching assays also showed that at all observations, the number of viable eggs was greater than the number of hatched juveniles. This suggests an inhibitory effect of anaerobic conditions created with inundation alone and in combination with amendments, on the hatching of J2 from viable eggs. Wharton and Perry (2011) stated that when nematodes are exposed to flooded and anaerobic conditions, they tolerate the hyposmotic and oxygen stress by entering states of quiescence, called osmobiosis and anoxybiosis. Spaull *et al*. (1992) reported that the hatch of *G. pallida* J2 declined after long periods (28-35 weeks) of exposure to anaerobic conditions. Wallace (1955) found a decline of the emergence of *Heterodera schachtii* J2 even after a short exposure (one day) to low oxygen concentrations. Van Gundy & Stolzy (1963) reported that the hatching of *Hemicycliophora arenaria* was reduced at low oxygen concentrations; however, the movement of the nematode was not influenced. In the pluronic gel assay, the host finding ability of

surviving J2 from cysts retrieved from the inundated soils was not influenced by the addition of amendments. Similarly, comparing the effects of non-amended soil and amended soils on the infectivity of J2 showed that their ability to penetrate the roots was not influenced by the amendments. This was the case in the infectivity assay on potato roots in sand and on tomato roots in pluronic gel. Nevertheless, there will not be any high risk of introduction of PCN when inundated treated waste soil is taken back to the field since no J2 hatched from eggs inside cysts recovered from inundated amended soil after 4 weeks.

In conclusion, the experiments undoubtedly showed that amendments speed up the disinfestation process of inundation by reducing the viability of PCN in soil. The spread of PCN can be avoided through the killing of eggs and consequent inhibition of the hatching of J2, a very important life stage as it infects the potato roots.

Obviously, the addition of agro-industrial waste products to inundated soil is an effective and environmentally friendly practice for disinfesting waste soil contaminated with PCN. Shortening the time of inundation allows faster disinfesting of industrial waste soil, thereby avoiding the accumulation of large quantities of soil. Amending soil with potato peels or green leaves of leek reduced the survival of PCN. The use of potato and leek waste for disinfestation of soil contaminated with PCN also disposes part of this organic waste produced in agricultural industries. It should be noted that the disinfestation of PCN-contaminated soil can only be effective when the average temperature is high enough to obtain an effective suppressive effect of inundation on PCN survival. Previous studies showed the influence of temperature on the effectiveness of inundation in the management of PCN; soil inundation at higher temperatures shortened the duration of inundation (Spaull *et al*., 1992; Runia *et al*., 2012). Therefore, in cold periods of year, this procedure will take longer.

Annually, an estimated 86000 tonnes waste soil is collected in the potato processing industries after about 3.8 million tonnes of potato is processed. 20-25 g organic waste products used in the laboratory experiments represent maximum 1720 to 2150 tonnes organic wastes required to disinfest the waste soil collected in the factories yearly. This amount is much less than the agro-industrial wastes produced in processing industries or leek fields every year. For example, the annual leek waste in Belgium after harvesting, cleaning and sorting, is estimated at

about 42000-63000 tonnes. The water collected in the factories after washing tubers can also be used for saturating the waste soil. The inundated treated soil can be taken back to agricultural fields and used as fertiliser. Since there is zero tolerance for PCN, the duration of inundation should be prolonged to 5 weeks to achieve 100% reduction in viability. As more attention needs to be paid to soil as an important means for spreading PCN and other soil-borne pathogens and weeds, more investigation is needed to apply the method in the processing industries in a suitable practical way. Recent studies on potato diseases showed a decrease of soil-borne pathogenic fungi such as *Ralstonia solanacearum* (van Overbeek *et al*., 2014) and *Sclerotinia sclerotiorum* (Niem *et al*., 2013) in flooded conditions, but not of *Verticillium dahliae* (Niem *et al*., 2013). Therefore, it is also necessary to identify the potential of increasing or decreasing pathogenic fungi or bacteria related to potato in treating waste soils with inundation.

Chapter 8

General discussion

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are the most problematic plant-parasitic nematodes of potato in Europe. They are classified as quarantine organisms in most countries where they can cause yield reduction of potato. Strict quarantine measures combined with other management strategies (*e.g.*, crop rotations, and the judicious use of nematicides and resistant cultivars) are in place to mitigate the introduction and eventual damage and spread of PCN (Anon., 2007; Suffert, 2014). This is of importance because once PCN becomes established, protection provided by the cyst wall and the eggshell reduce the efficacy of many management strategies. The ability of PCN to be in a dormant state and survive between successive host crops until hatching of second-stage juveniles (J2) once host plants are present, contribute significantly to PCN survival (Evans & Perry, 1976; Perry, 1989). Hence, any management strategy that influences the viability of unhatched J2, their hatching, mobility, host finding ability, and invading the host roots, can effectively lower the PCN populations in the field.

Survival of PCN can be affected by several factors, *e.g.*, soil temperature (Stone & Webley, 1975; Boen *et al*., 2006), soil moisture (Spaull *et al*., 1992; van Overbeek *et al*., 2014) and soil oxygen (Wallace, 1955). Therefore, manipulating soil or agricultural practices to create an adverse condition for PCN will impact their survival and improve the effectiveness of management strategies.

It has been shown that very early harvesting of early potato cultivars decreases PCN populations (Webley & Jones, 1981). Early harvesting curtails the growing period at low soil temperatures thereby reducing the time for the nematodes to complete their life cycle and to produce cysts. In Belgium, the legislation allows farmers to grow potatoes in rotations shorter than three years, provided the tubers are harvested before 20 June (Anon., 2010). This policy is based on the assumption that no cyst formation is completed by that date because of the low temperatures during a short growing period. However, considering the global climate change, this assumption might not be valid anymore. To verify the validity of this ultimate harvest date, I determined the duration of the life cycle of *G. rostochiensis* and *G. pallida* in the field, in microplots and in the growth chamber under temperature regimes similar to those in the fields (Chapter 4). I also investigated the relationship between the heat accumulation and the development of both PCN species as well as the influence of the potato cultivar and the

population of PCN on the time required for cyst development. Despite the low soil temperature and the short growing season, both PCN species completed their life cycle before 20 June, irrespective of the nematode population and the potato cultivar. Chavornay, the virulent population of *G. pallida*, did not hatch earlier nor did it develop any faster than the local population GOV 038. The occurrence of *G. pallida* stages on susceptible potato cultivars was also similar. For both PCN species, the final population density was higher than the initial populations (*P*f > *P*i). Fewer heat units were needed for the development of *G. rostochiensis* than for *G. pallida.* Population dynamics in relation to degree-day accumulation have been considered in previous research (Greco *et al.*, 1988; Alonso *et al.*, 2011). Females of PCN were observed at degree days close to those observed in my study. No females of *G. pallida* were detected on the roots of potato cv. Ambassador (partial resistant to this species). The PCN species showed a substantial difference in hatching and temperature responses. Low soil temperatures favoured *G. pallida* that emerged earlier at lower soil temperatures (8.8-10°C *vs* 10.1-11°C) and showed a higher multiplication rate than *G. rostochiensis* (9.2-12.0 *vs* 2.9). Previous research revealed differences in temperature requirements between these PCN species (Foot, 1978; Webley & Jones, 1981; Kaczmarek *et al*., 2015). Both, Robinson *et al.* (1987a) and Kaczmarek *et al*. (2015) reported optimum thermal conditions for hatching to be lower for *G. pallida* than for *G. rostochiensis*. This difference might eventually influence the competition between co-existing *G. rostochiensis* and *G. pallida* in the field at different soil temperatures as well as their final proportions in the field. Both PCN species completed their life cycle at suboptimal and low soil temperatures with a relatively high multiplication rate. Obviously, considering the increase in temperature, the maximum population levels of PCN and the damaging levels can be reached faster and eventually more challenges for the PCN management are to be expected. Usually there is only one generation per potato crop, but there is evidence that a second generation of PCN can occur under certain environmental conditions leading to a higher reproduction rate (*e.g*., Evans & Stone, 1977; Greco *et al*., 1988; Jimenez-Perez *et al*., 2009; Kaczmarek *et al*., 2015). No second generation of PCN was recorded throughout my experiments. However, it merits further investigation under Belgian climate conditions in both early and late potato growing regions.

Amending field soil with organic materials as fertilisers (*e.g*., animal manures) is a common practice by potato growers in Belgium. These amendments not only maintain the soil quality and fertility by improving the biological, physical and chemical properties of the soil

(Efthimiadou *et al*., 2010; D'Hose *et al*., 2014), but also reduce plant pathogens including nematodes (Renčo *et al*., 2007; Oka, 2010). Cysts of both PCN species were exposed to several amendments, *e.g*., pig slurry, cattle slurry, mineral nitrogen fertiliser (NH_4NO_3), crab shell compost, wood chip compost, biochar (Romchar) alone and in combination with wood chip compost, pig slurry or crab shell compost. The effects of these amendments were studied in pot experiments on the viability of encysted eggs and J2, as well as on the survival of hatched J2 and their reproduction on potato (Chapter 6).

All amendments mixed with the soil, apart from biochar (Romchar), increased the mortality of encysted eggs and J2, and inhibited subsequent hatch of J2 in the absence of potato. However, the protection of unhatched J2 by the eggshell and the cyst wall seems to influence the effectiveness of the amendments to control PCN. Valdes *et al*. (2011) did not observe any little effect on unhatched PCN J2 of extracts from green manures, suggesting a protective role of the cyst wall and the eggshell. Yet, it should be noted that the integration of the soil amendments between successive crops (no potato cropping) will still increase the population decline rates; 22% and 25% reduction of viability was observed for *G. rostochiensis* and *G. pallida*, respectively. *In vitro* assays provided more insight on the value of amendments for PCN management. Combining amended soil with potato was more effective against PCN than the sole incorporation of amendments. The mechanisms of nematode suppression differed as a function of the amendments. Some amendments such as animal manures, crab shell compost and nitrogen fertiliser, caused a significant reduction of the hatching, migration and infectivity of J2. Therefore, the incorporation of these amendments at planting appears to be a good practice to lower the population of PCN. Wood chip compost stimulated the hatching of PCN in the presence of the host. This suggest that planting a PCN-resistant potato cultivar in in soil simultaneously amended with wood chip compost and planted to potato, results in increasing J2 hatch while their multiplication would be prevented or reduced by resistance. Ammonium and volatile fatty acids are generally ruled out for their involvement in nematode suppression in amended soils with organic materials (Chitwood, 2002; Oka, 2010). The production of volatile fatty acids was not determined in my study. However, the generation of ammonium in soil amended with nitrogen fertiliser, pig and cattle slurry and changes in the microbial community of amended soil in my trials might be the most reliable mechanisms of PCN suppression.

A major means of spread of PCN is the transportation of soil contaminated with cysts, either attached or not to potato tubers taken from the fields (Goeminne *et al*., 2011; EFSA, 2012). Effective disposal of this waste soil is an important component of any PCN control program. The survival of the eggs and J2 of PCN cysts was examined in waste soil from potato processing industries, after being inundated and exposed to agro-industrial waste products, *viz.* potato peels and green leaves of leek (Chapter 7). Application of agricultural and industrial waste amendments clearly improved the effect of inundation on the survival of PCN. Inundation of waste soil amended with industrial waste products mentioned above for 28 days caused almost complete death of the cysts content. Potato peels caused a significant reduction in the pH and the redox potential of soil. A considerable increase in the concentration of short chain fatty acids (SCFA) was also observed in soil amended with potato peels. The phospholipid fatty acid (PLFA) analysis also showed an increase in the total biomass of the microbial community of soils amended with potato peels. However, the PLFA analysis did not identify the microorganisms in anaerobic conditions at higher taxonomic levels to allow better understanding of the suppression mechanisms involved. Therefore, the depletion of oxygen, reduction in pH, the formation of SCFA and increase in total biomass of microbial community in inundated amended soils with potato peels can be related to nematode suppression.

Amending soil with leek also caused a significant reduction in pH and the redox potential of soil. However, no increase was observed in the total biomass of microbiota. There was only an increase in gram negative bacteria. The high amounts of short chain fatty acids and H_2S were not always detected in the leek amended soil; this is probably due to the variability in the different batches of leek used in different experiments or the time when the gas measurement was performed. Nevertheless, the PCN viability was still reduced in soil amended with leek without the detection of H_2S and high amount of SCFA. Therefore, the depletion of oxygen and reduction in pH might be the major factors causing the egg mortality. Previous studies also reported an effective suppression of PCN in inundated conditions (Spaull *et al*., 1992; Runia *et al*., 2012; van Overbeek *et al*., 2014). Production of hydrogen sulphide (H_2S) (Spaull *et al*., 1992) and the generation of SCFA in inundated conditions (Runia *et al*., 2012) were suggested to be responsible for the mortality of *G. pallida* in such circumstances.

These results suggest that soil disinfestation using waste products of the processing industries is an effective and environmentally safe way to disinfest the waste soil that can contribute to the recycling of waste by-products of the agro-industry. The application of this method can probably be justified economically in terms of the cost of construction of a reservoir to subject the soil to inundation in the potato processing industry (Van Lembergen *et al*., 2010). Further research is required to validate the efficacy of this strategy when used in industry. These studies would need to be extended over several seasons to assess the effectiveness of inundation of amended soil in different temperature conditions. It is also worth exploring the effect of this method on the survival of other plant-parasitic nematodes associated with potato, *e.g*. *Meloidogyne* spp. in waste soil, debris and plant materials. The disadvantages of using inundated soil include the possibility of introducing resilient pathogens, as well as changes in structure, fertility and pH of the soil. Therefore, further research should also focus on the identity of the soil microorganisms involved and on the soil quality after flooding the amended soil.

The integrated management of PCN relies heavily on information regarding the soil population densities at planting time (Seinhorst, 1967), taking into account the viability of the eggs inside of the cysts. The egg viability of PCN can be measured by visual assessment, Meldola's blue staining, hatching assays, or plant infectivity assays (OEPP/EPPO, 2013). However, these assays contain an element of subjectivity or are labour intensive and time consuming. For these reasons, I optimised a method developed by van den Elsen *et al.* (2012) that determines the number of viable eggs of PCN based on the trehalose present in live eggs. The trehalose-based method yielded data on egg viability that were comparable with the viability determined by visual assessment, which, however, is time consuming, requires trained personnel, and can involve some subjectivity. The trehalose-based method proved to be an objective, sensitive, reliable, robust, fast, and cheap technique for assessing the number of viable eggs in PCN cysts. The method was used to assess the viability of encysted eggs exposed to amendments in pot experiments (Chapter 6) and in inundated conditions (Chapter 7), and the results were satisfactory. It is worth exploring the possibility of using this method to assess the viability of eggs of other cyst nematodes such as *Heterodera* spp.

Overall, the work outlined in this thesis has demonstrated that cultural management strategies, *e.g*., early harvesting can have a significant suppressive effect on the PCN population

build-up in the field. The addition of agro-industrial products to soil have also the potential to reduce the survival and reproduction of PCN in the field, as well as in waste soil contaminated with the PCN cysts. However, the effects of amendments should be investigated in the field on soil-plant-nematode systems through long-term experiments.

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